Canadian Journal of Research

Issued by The National Research Council of Canada

VOLUME 5 SEPTEMBER, 1931 NUMBER 3

HEIGHT OF THE POLAR AURORA IN CANADA[1]

By J. C. McLennan[2], F.R.S., H. S. Wynne-Edwards[3] and H. J. C. Ireton[4]

Abstract

Simultaneous photographs of the polar aurora were made at two stations in northern Ontario, Canada, and from them a number of values for the height and position were obtained. For the lower limits of arcs and bands these ranged between 70 and 130 km. with a maximum frequency between 90 and 95 km. The mean height for all such lower limits was 95 km. The distribution of the points with reference to the earth's surface is shown.

Introduction

The aurora borealis has been the subject of many researches, particularly by Norwegian scientists. Professor Størmer* has made a long series of observations on the distribution, form and height of the aurora and its dependence on concomitant phenomena, and has developed the method of height measurement by simultaneous photographs on an extended base. While many of his results are of undoubted generality, it is nevertheless important that data should be obtained in as many other northerly regions as possible.

Canada, and particularly northern Ontario, is very favorably situated for such investigations. Owing to the position of the magnetic pole the zones of corresponding auroral activity lie further south there than elsewhere in the northern hemisphere, so that the aurora is commonly seen in lower latitudes than in Europe. For these reasons it was decided to make observations at a point as far north as is compatible with reasonable facilities.

Observation Stations

The main observing station was situated at Onakawana on the northern extension of the Temiskaming and Northern Ontario Railway at present under

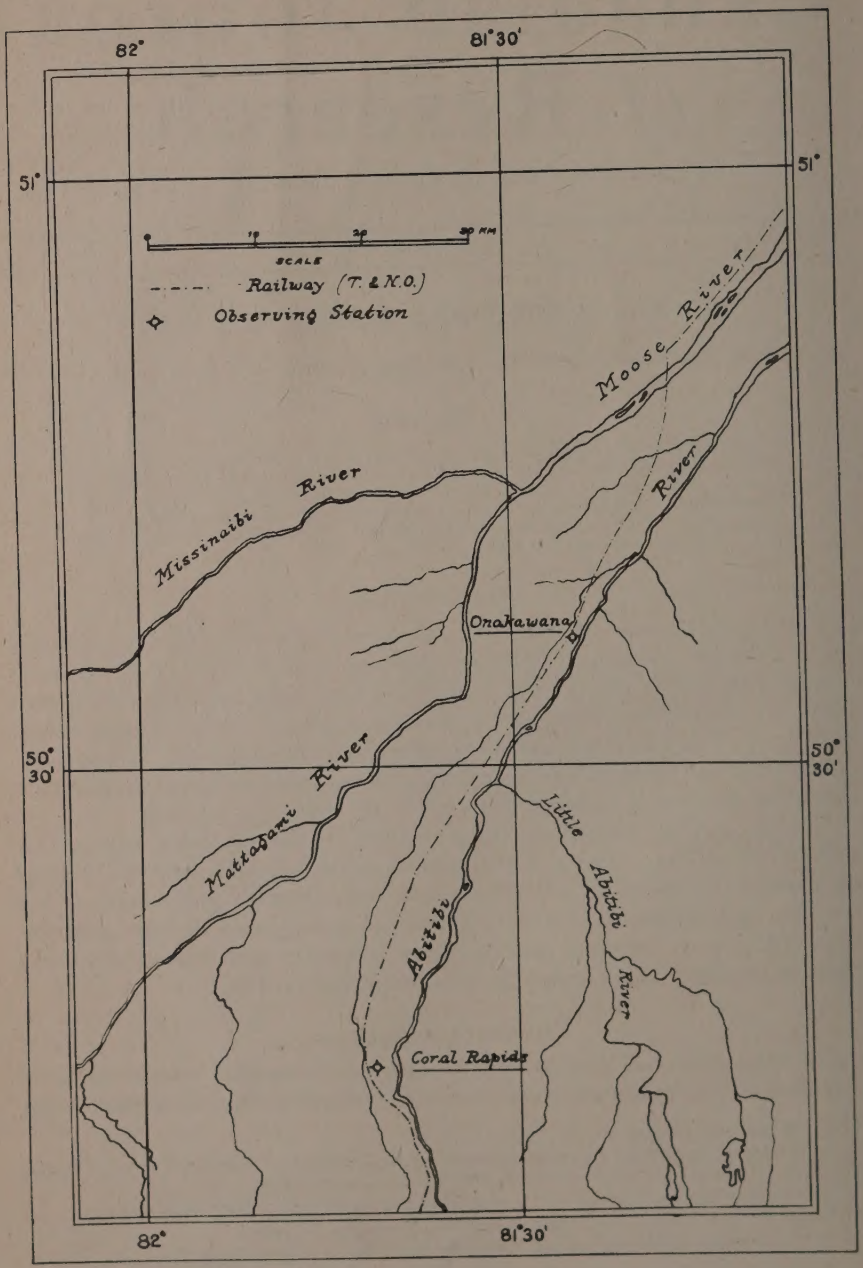
[1] *Manuscript received May 30, 1930.*
Contribution from the Physical Laboratory of the University of Toronto, Toronto, Canada, with financial assistance from the National Research Council of Canada.

[2] *Professor of Physics and Director of the Physical Laboratory, University of Toronto.*

[3] *Research Assistant, Department of Physics, University of Toronto.*

[4] *Lecturer and Research Associate, Department of Physics, University of Toronto.*

**Résultats des mesures photogrammétriques des aurores boréales observées dans la Norvège méridionale de 1911 à 1922, Geofysiske publikationer, Vol. IV, No 7, Oslo 1926, in which references to other publications may be found.*



MAP 1. *Map showing location of observation stations.*

construction, and here a small laboratory was equipped. The substation was at Coral Rapids, where photographs were taken by Mr. T. J. Kennedy, and the two stations were connected by the railway company's telephone line.

The length and azimuth of the base line were determined from the plans of the railway right-of-way, and the latitude and longitude of Coral Rapids from the survey of the Department of Lands and Forests of the Provincial Government.

Length of base line, b	= 46.40 km.
Azimuth of Onakawana from Coral Rapids, A_0	= N 22°31′ E.
Latitude of Coral Rapids, ϕ	= 50°13′ N.
Longitude of Coral Rapids, λ	= 81°40′ W.

The heights of the two stations above sea level were approximately 50 metres for Onakawana and 110 for Coral Rapids, and the height-angle involved has been considered negligible.

Apparatus and Method of Observation

The cameras used were of a type designed by Professor O. Krogness of Tromsø, and arranged so that by moving the lens to different positions six small photographs may be taken on one 9 by 12 cm. plate. The lenses were Kino-Plasmat, D.R.P. Dr. Rudolph, made by Messrs. Hugo Meyer of Görlitz, with aperture 1.5 and focal length, 5 cm. The plates used were Herzog Sonia E W.

In order to synchronize the exposures the two observers used operator's telephones which could be worn without interfering with their movements. The time was taken from an accurate watch which was checked daily by radio signals. Since there was only one person at each station it was impossible to avoid taking the watch into the cold, but it was protected as much as possible from temperature changes.

The plates taken at Coral Rapids were sent as soon as means were available to Onakawana, and all the plates were developed there, corresponding plates being developed at the same time.

Method of Calculation

In computing the auroral heights and distances, one of each pair of simultaneous pictures was projected by a lantern on a ground glass screen, and as many stars and well-defined auroral points as possible traced on the latter. The second photograph of the pair was then so arranged in the lantern that the projected stars coincided with those already drawn on the screen, and the auroral points corresponding to those selected in the first plate were marked. In nearly every case a sufficient number of stars could be identified to admit of the insertion on the projection of the declination and right ascension circles of the celestial sphere and from this co-ordinate system a slight extrapolation enabled the declination δ and right ascension α of the auroral points to be read off. If δ_1, α_1; δ_2, α_2 are the co-ordinates for two corresponding auroral points, their angular separation, *i.e.*, the parallax of the point in question on the aurora, is given by the standard formula:

$$tg\ p = \frac{\alpha_1 - \alpha_2}{\delta_1 - \delta_2} \cos \frac{\delta_1 + \delta_2}{2} \qquad (1)$$

which is valid for such small separations as were most frequently observed. In cases where so few stars could be distinguished as to preclude a reasonably accurate insertion of a co-ordinate system, the scale of the projected diagram in the vicinity of points of interest was calculated from pairs of stars, their angular separation being computed by (1) (or by a more accurate formula if the separation is larger), and the linear separation of the auroral points was then turned into angular measure.

From the sidereal time of exposure the right ascension of an auroral point δ, α can be expressed as an hour angle t, and by the standard equations of Gauss we may calculate the altitude h and azimuth A of the point:

$$\sin h = \sin \phi \sin \delta + \cos \phi \cos \delta \cos t \quad (2)$$

$$\sin A \cos h = \sin \delta \cos t \quad (3)$$

where ϕ is the latitude of the station. From the parallax p, the length b of the base line and its direction A_0 a simple right-angled spherical triangle alone remains to be solved to give the angle u between the base line and the line from the observer to the auroral point. We now compute the quantities

$$r = b \sin u / \sin p$$

$$x = r \cos h$$

$$y = r \sin h$$

and hence the height H of the auroral point and the distance D to the point vertically below it;

$$H = \sqrt{(R+y)^2+x^2} - R$$

$$D \doteqdot \frac{R}{R+H} x$$

where R is the radius of the earth. We have neglected the height of the stations above sea level.

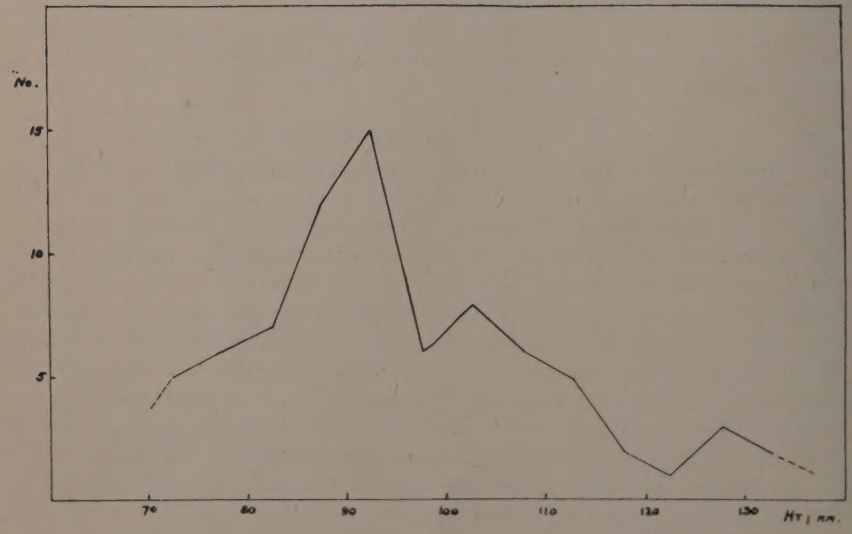


FIG. 1. *Relation between heights and frequency of occurrence.*

It will be seen that the method is essentially the simple one of Størmer used in his early work of 1912-1913. Størmer's later method involving the construction of nets to correct for distortion when a portion of a sphere is projected on a plane is unnecessary here; the smallness of h for example would hardly justify the more elaborate process. The results obtained vary in accuracy on account of several factors, the chief of which is the possibility of recognizing corresponding auroral points. In the favorable cases, however, the height will not be more than 5% in error; in about one-fifth of the cases it may be as great as 10%, and in very unfavorable cases may exceed 20%.

Results

Diagrammatic sketches of all the photographs measured are given in Fig. 2-4, in which the auroral outlines and points treated, together with their parallaxes, are qualitatively indicated. All the points lie on the lower limits

TABLE I

No.	Date 1931	Time			Exposure, sec.	Reference stars
		hr.	min.	sec.		
3	Jan. 25	23	26	30	60	α, β, η Cas, ϕ, ν And
4		23	30	35	60	α, η, ζ Boo, α, C Vn
5		23	33	10	30	α Dra, Θ, γ Boo, ζ, ε, U Ma
6		23	34	52	15	ε Vir, α, ε, η Boo
7		23	37	40	10	α, β, ζ, o Cas, κ And
8		23	39	47	5	β Cas, κ, o And, 2 Lac
9		23	41	55	27	β, ζ Cas, κ, o And
10		23	42	30	32	κ, o And, π^2 Cyg, ξ Cep
13		23	48	42	5	α, o And, α Lac, β, ζ Cas
14		23	50	10	19	α, β, γ, δ, ζ Cas
16		23	54	00	51	α, γ, δ, η, μ Boo
17		23	56	10	37	α, γ, λ Boo, α C Vn
19	Jan. 26	0	02	15	8	α, γ, λ Boo, η, ζ U Ma
20		0	03	55	55	α, ε, η, τ Boo
21		0	06	20	60	ε Vir, α, ε, η Boo
22		0	18	02	25	α Cr B, β, γ, δ, ε, λ Boo
23		0	20	15	90	α Cr B, α, η Boo
24		0	24	11	48	β, γ, δ, ν, π, ξ Dra
25		0	27	15	57	β, γ, ν, ξ Dra, τ Her
27		0	31	15	35	41 Ari, β Tri, β, γ And
28		0	32	38	25	β, ν, ξ Dra, τ Her
29		0	33	10	40	β, γ, ν, ζ Dra, τ Her
30		0	35	50	30	α, λ, ξ Tau, 41 Ari, ζ, ξ Per
31		1	11	30	80	α, β, γ, ζ Cas
32		1	13	05	17	α, β, γ, ζ Cas
33		1	13	30	10	41, 35 Ari, γ, β Tri
34		1	16	17	4	η Tau, β, ζ Per
36		1	21	05	48	β, γ, ε Boo, γ, τ, ϕ Her
37	Jan. 27	21	39	19	30	β, η Peg, o And, α, π^2 Cyg
38		21	41	41	40	ε, η, ζ U Ma, α C Vn, γ Boo
39		21	44	43	33	β, γ, ν, ξ, η, ϕ, Dra, β, γ U Ma
40		21	49	47	24	α Cep, α Cyg, α Lac
41		21	52	04	18	α Cyg, α, 4 Lac, α, β Cep
43	Feb. 13	22	22	05	19	γ, β, ξ, ν, η Dra, τ Her
44		22	23	50	44	τ Her, γ, β, ν, ξ, 39 Dra
45		22	26	17	5	β, γ, ν, ζ, ξ, η, Θ Dra
46		22	27	40	65	α Cyg, α, β Cep, δ Dra
47		22	28	30	14	α, β, γ, ζ Cas, γ And
48		22	30	36	6	α, β, γ, ζ Cas

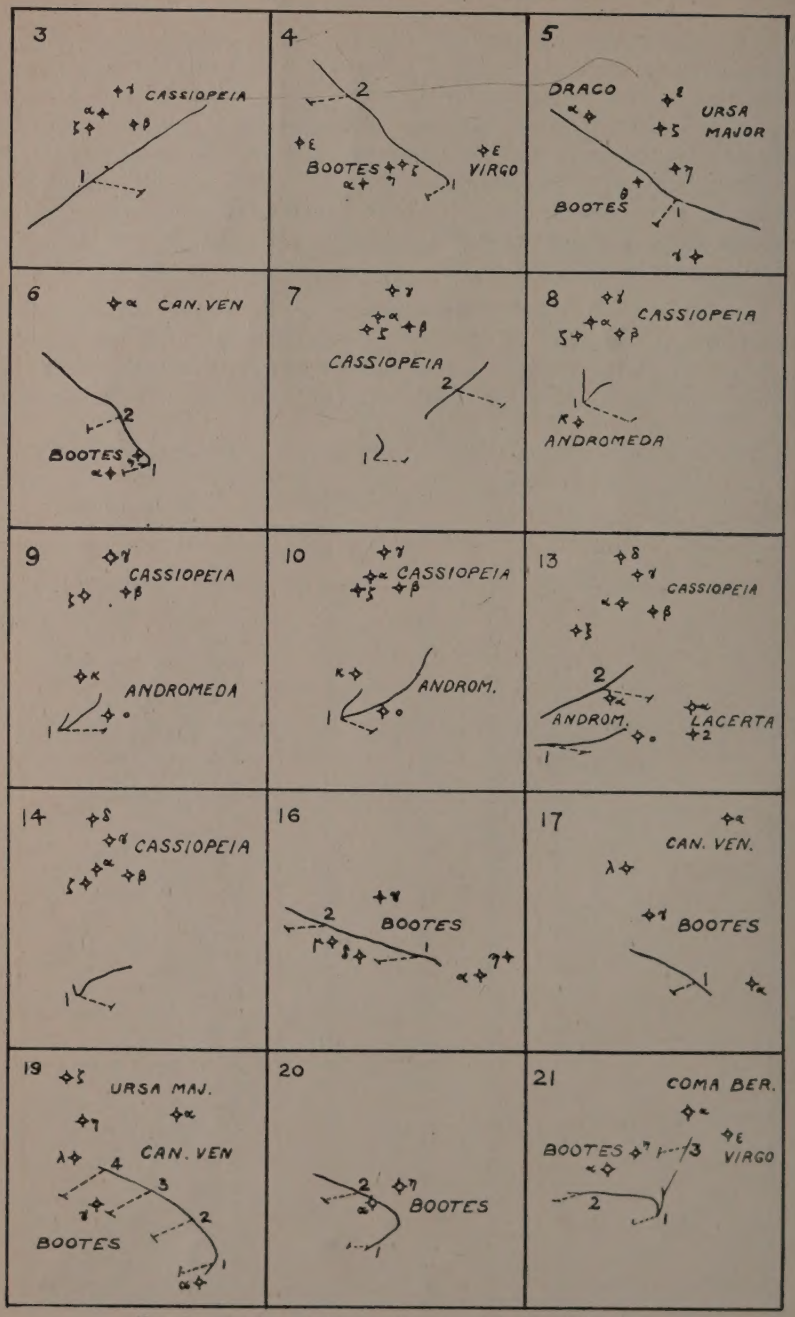


FIG. 2. *Sketches of photographs measured: No. 3-17 taken Jan. 25, 1931; No. 19-21 taken Jan. 26, 1931.*

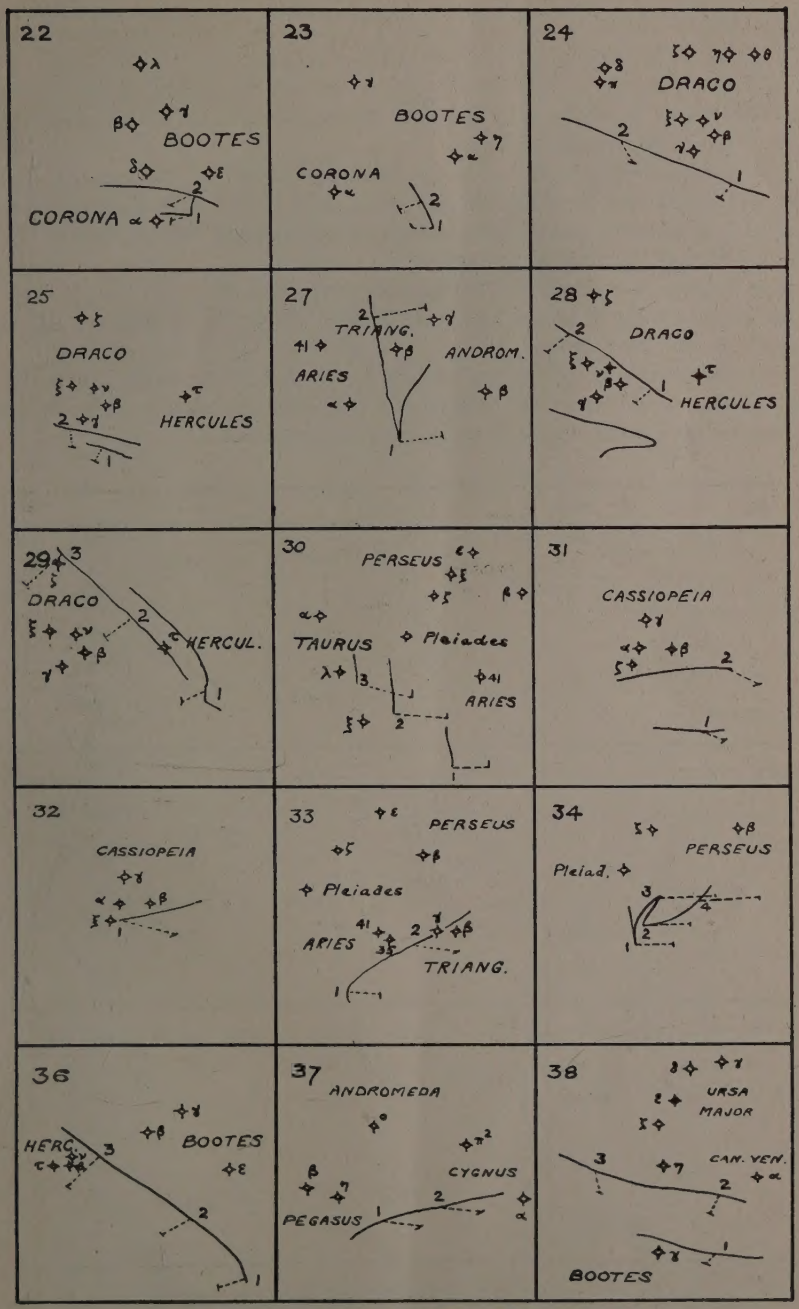


FIG. 3. *Sketches of photographs measured: No. 22-36 taken Jan. 26, 1931; No. 37 and 38 taken Jan. 27, 1931.*

of bands with the exception of No. 21,3; 27,2 and 48,2 which lie on streamers, and of No. 39,5 which is on the upper limit of a band.

In Table I are given the 75th meridian time at the middle of the exposure and the length of exposure. The fifth column shows the principal reference stars used in the measurements.

The results of the measurements are shown in Table II. In it are tabulated the parallactic angle p of the auroral point, α, δ, A and h, its right ascension, declination, azimuth and altitude with reference to Coral Rapids, and H and D the height and distance of the point.

The heights have been plotted against frequency of occurrence in Fig. 1, those heights lying in ranges less than 70, from 70 to 75, 75 to 80, ..., km. being collected into groups.

The distribution of the auroral points in relation to the earth's surface has been indicated on Map II. Points on the same definite band have been joined, isolated small circles showing single observations.

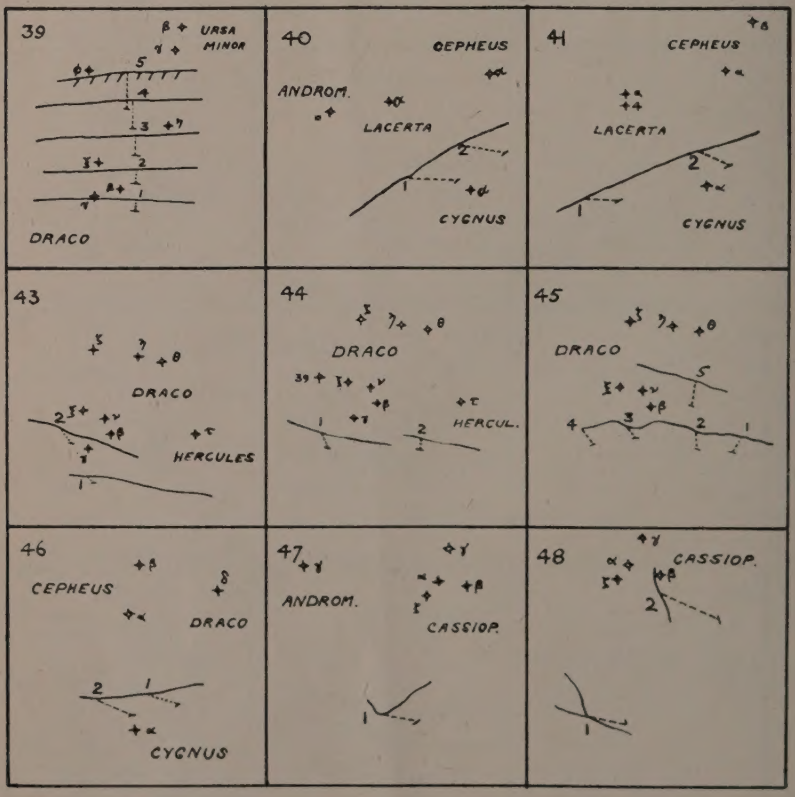
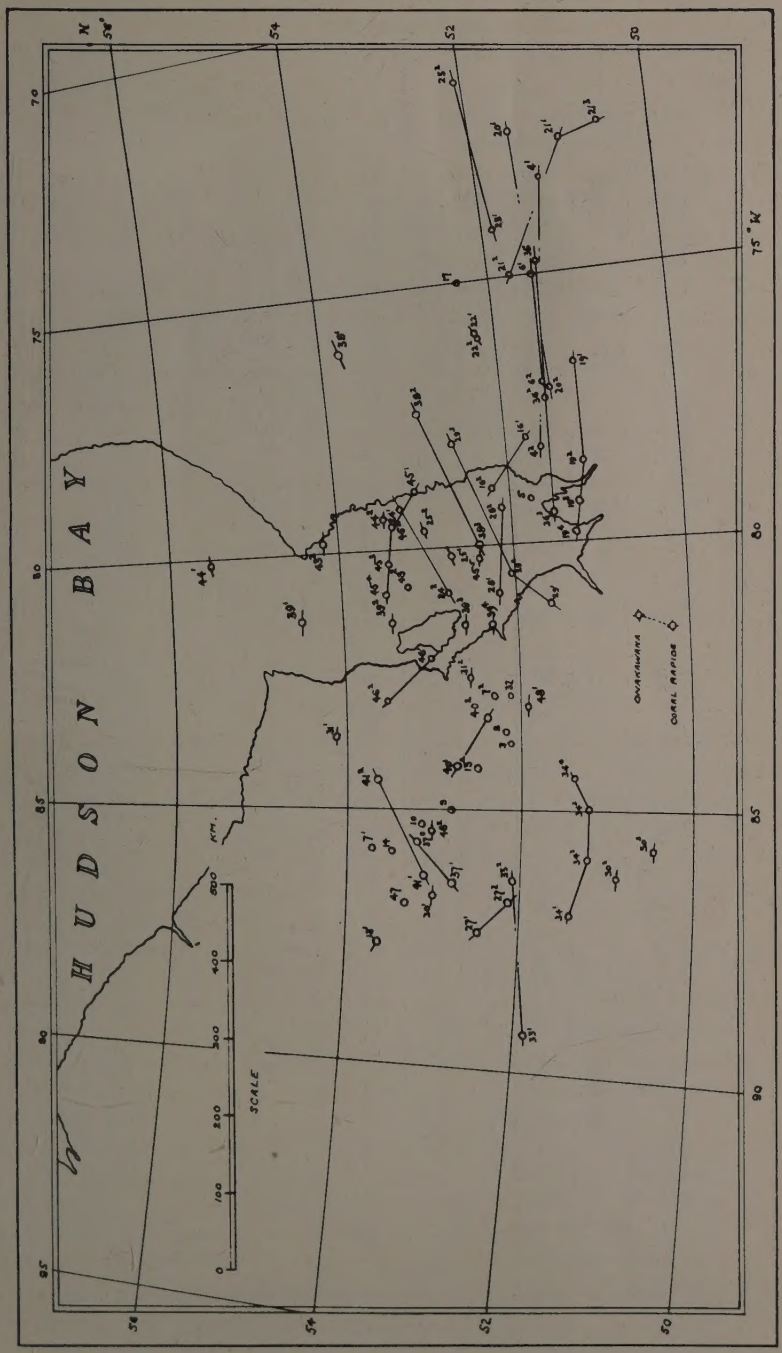


FIG. 4. *Sketches of photographs measured: No. 39-41 taken Jan. 27, 1931; No. 43-48 taken Feb. 13, 1931.*



MAP 2. *Distribution of auroral points in relation to the earth's surface.*

TABLE II

No.	Date 1931	Time			P	p °	α		δ °	A−180° °	h °	H km.	D km.
		hr.	min.	sec.			hr.	min.					
3	Jan. 25	23	26	30	1	8.7	23	01	51.4	−33.8	22.1	103	239
4		23	30	35	1	3.6	13	42	12.3	78.3	6.3	96	603
					2	5.8	14	02	34.7	59.7	20.2	111	280
5		23	33	10	1	5.8	14	37	45.7	46.8	24.3	109	228
6		23	34	52	1	3.9	14	03	18.0	71.5	8.0	93	486
					2	5.1	14	01	27.0	65.9	15.2	108	354
7		23	37	40	1	4.6	22	41	41.5	−34.5	10.9	111	474
					2	7.7	21	38	56.2	−18.4	19.7	86	226
8		23	39	47	1	8.7	22	46	50.3	−30.5	18.8	85	234
9		23	41	55	1	6.2	22	58	38.3	−38.3	9.4	71	365
10		23	42	30	1	5.8	22	56	39.8	−37.0	10.4	84	390
13		23	48	42	1	4.5	23	31	33.8	−45.3	8.3	101	530
					2	7.4	23	10	47.1	−34.5	17.3	99	290
14		23	50	10	1	5.0	23	02	39.3	−37.1	9.7	93	446
16		23	54	00	1	5.1	14	56	30.4	57.3	12.7	77	304
					2	3.9	15	41	42.3	42.5	16.7	93	283
17		23	56	10	1	3.2	14	50	26.3	61.5	10.8	122	518
19	Jan. 26	0	02	15	1	5.7	14	19	17.5	74.0	9.5	71	359
					2	7.7	14	13	28.5	67.6	18.7	87	239
					3	8.2	14	26	36.4	59.7	22.6	89	202
					4	8.6	14	46	43.0	51.7	24.8	81	169
20		0	03	55	1	2.9	14	39	15.8	74.5	5.3	101	680
					2	5.3	14	32	24.8	67.0	13.3	92	344
21		0	06	20	1	3.3	14	18	10.0	79.8	4.5	88	661
					2	3.8	14	41	20.3	68.8	8.9	98	494
					3	3.3	13	32	16.2	84.4	16.5	244	674
22		0	18	02	1	3.6	15	23	25.8	59.9	8.8	86	448
					2	3.6	15	16	27.3	60.2	10.9	103	445
23		0	20	15	1	2.6	15	00	15.5	71.0	4.4	105	753
					2	3.4	15	00	18.6	69.0	6.8	93	559
24		0	24	11	1	1.3	17	43	44.2	26.8	10.1	77	368
					2	2.9	18	54	53.3	12.2	15.0	80	273
25		0	27	15	1	1.5	17	54	45.5	24.8	10.7	72	329
					2	2.0	18	08	48.6	21.4	12.7	76	285
27		0	31	15	1	5.6	1	00	27.8	−56.9	9.1	90	455
					2	5.8	2	00	38.3	−59.7	25.2	207	399
28		0	32	38	1	3.0	17	17	48.3	30.3	16.4	84	261
					2	4.6	18	32	59.9	14.8	22.5	93	213
29		0	33	10	1	2.9	16	29	38.0	43.7	12.3	92	365
					2	4.6	16	57	53.0	30.8	22.1	90	210
					3	7.6	18	00	64.4	16.8	28.0	81	146
30		0	35	50	1	5.4	2	07	16.5	−46.5	9.5	93	453
					2	7.7	2	46	18.2	−75.2	16.9	108	323
					3	8.4	3	13	17.2	−81.7	20.4	115	285
31		1	11	30	1	3.7	22	36	48.8	−15.4	11.0	104	445
					2	5.8	22	13	59.5	− 9.4	20.6	103	256
32		1	13	05	1	8.3	23	27	57.2	−19.8	21.3	88	214
33		1	13	30	1	4.7	2	21	20.0	−68.9	8.5	109	552
					2	6.7	1	57	33.2	−56.1	15.3	115	373
34		1	16	17	1	6.6	2	43	20.8	−69.6	14.5	113	386
					2	8.2	2	44	25.8	−68.7	15.8	96	308
					3	9.7	2	41	30.2	−65.0	18.7	94	257
					4	10.4	2	14	35.3	−57.0	18.8	86	226
36		1	21	05	1	4.1	15	43	16.0	74.0	7.6	87	500
					2	5.3	15	49	28.0	65.0	15.8	104	335
					3	6.7	16	18	43.4	49.5	23.5	94	207
37	Jan. 27	21	39	19	1	5.3	21	43	34.4	−47.4	10.4	94	427
					2	5.4	21	08	41.3	−37.6	12.4	107	418
38		21	41	41	1	1.8	14	01	35.0	42.7	8.1	102	545

TABLE II—*Continued*

No.	Date 1931	Time hr.	Time min.	Time sec.	P	p °	α hr.	α min.	δ °	A−180° °	h °	H km.	D km.
					2	2.6	13	36	40.4	43.8	15.3	131	418
					3	3.0	14	52	51.4	26.1	17.8	91	262
39		21	44	43	2	2.1	17	15	53.5	3.9	13.9	136	469
					3	3.1	17	13	58.0	3.8	18.3	129	352
					4	4.5	17	11	61.7	3.8	22.0	111	258
					5	5.6	17	09	66.0	3.5	26.4	114	222
40		21	49	47	1	6.8	20	58	45.7	−32.0	14.3	87	308
					2	7.5	20	25	52.1	−23.9	17.6	86	252
41		21	52	04	1	5.3	21	35	36.5	−42.6	9.8	93	445
					2	4.7	20	30	49.5	−25.5	15.3	129	414
43	Feb. 13	22	22	05	1	1.1	17	56	46.5	15.8	8.7	86	450
					2	2.2	18	12	52.8	11.7	14.3	95	332
44		22	23	50	1	1.2	18	26	49.0	10.6	10.2	134	583
					2	1.1	17	08	43.8	25.1	9.0	72	378
45		22	26	17	1	1.6	16	40	41.2	31.5	9.0	67	355
					2	1.3	17	03	44.5	26.2	10.2	79	373
					3	1.6	17	53	48.8	16.5	11.3	82	356
					4	2.3	18	32	49.7	10.0	10.8	79	357
					5	3.0	16	50	50.8	25.5	16.8	80	246
46		22	27	40	1	4.3	20	05	50.5	− 5.0	11.1	67	300
					2	4.3	20	51	48.0	−12.9	9.7	74	366
47		22	28	30	1	4.9	23	34	37.4	−44.6	12.2	126	481
48		22	30	36	1	5.7	22	59	38.3	−37.9	9.0	77	397
					2	9.5	22	42	58.9	−25.7	24.9	98	200

Discussion of Results

It has not been considered useful to attempt a definite classification of auroral types. Owing to the southerly position of the stations and the lack of displays of particular ntensity during the past winter, practically all those seen were of the arc or band type, streamers or draperies being only rarely observed. The displays followed a very regular course which it may be of interest to sketch. As soon as darkness set in a faint glow was visible in the northern sky. This was never absent on any occasion when the sky was clear. Later on a single arc might appear at a higher altitude, and as a rule was quite steady in its position. The period of maximum activity was between ten and eleven o'clock, when intense displays might flash up very suddenly, generally starting as single or multiple arcs of a pulsating type from which streamers sometimes evolved. After a time the lights lost intensity and at the same time stretched further towards the zenith, until in the early morning they might extend some way towards the south, but too dim to be photographed.

It may be observed in parenthesis that among those who have spent some time in the north there is a general conviction that there is a noise associated with brilliant northern lights, but none was noticed in the course of these observations. First-hand evidence was also received of lights occurring apparently very low down, but of a nature to preclude definite conclusions.

The height measurements obtained are in good general agreement with the prevoius work of Størmer and others in Scandinavia. For the lower limits of

bands they show a range from approximately 70 to 130 km., with a marked maximum between 90 and 95 km. (see graph). The mean height for all lower limits of bands is 95 km. This is considerably lower than in Scandinavia, where the corresponding value obtained is about 105 km. Making allowance for experimental error it appears also that the lower limit is sometimes below the lowest height reached there, which Størmer gives as 80 km.

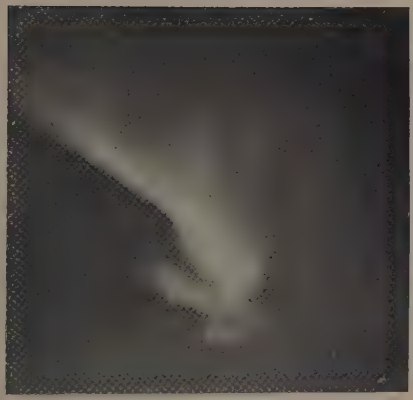
The material collected for other types of aurora is too limited to permit of any conclusions being drawn.

Acknowledgments

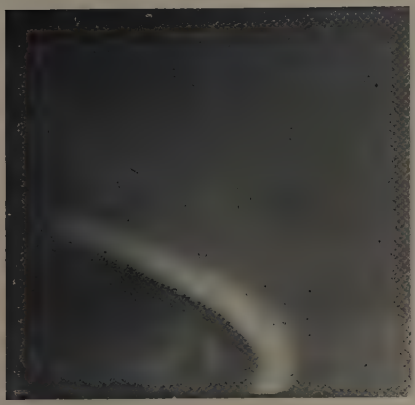
The authors are indebted to the Department of Mines of the Ontario Government for making it possible for one of them to spend several months at the Onakawana Coal Development fields, and to the T. and N.O. Railway for facilitating transportation and permitting the use of their telephone system. They wish to thank also the H. F. McLean Construction Company for their kindness in building the observing station at Onakawana, and the Bell Telephone Company of Canada for the loan of telephone equipment. The authors wish also to thank Mr. C. Barnes for developing a method for the rapid evaluation of results and the calculations connected therewith.



C 6



O 6



C 19



O 19



C 27



O 27

Typical simultaneous photographs of aurora taken at Coral Rapids (C) and Onakawana (O): No. 6 taken Jan. 25, 1931; No. 19 and 27 taken Jan. 26, 1931.



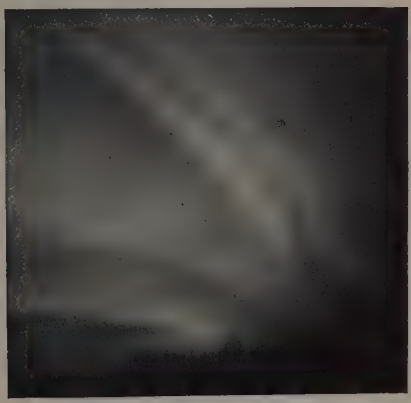
C 28



O 28



C 29



O 29



C 33



O 33

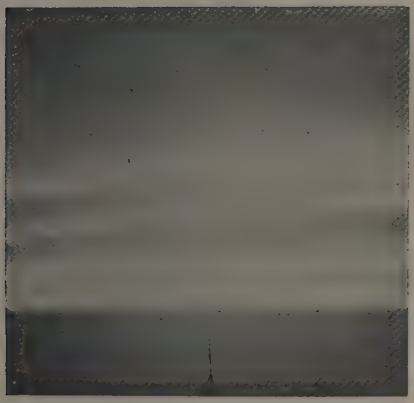
Typical simultaneous photographs of aurora taken at Coral Rapids (C) and Onakawana (O): all taken Jan. 26, 1931.



C 34



O 34



C 39



O 39

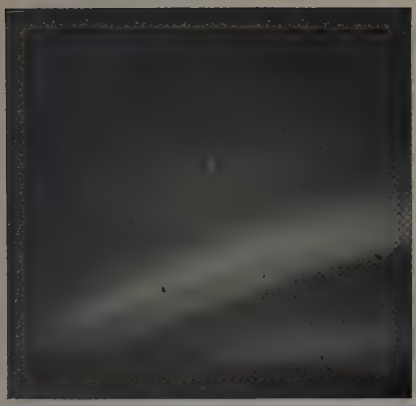


C 40

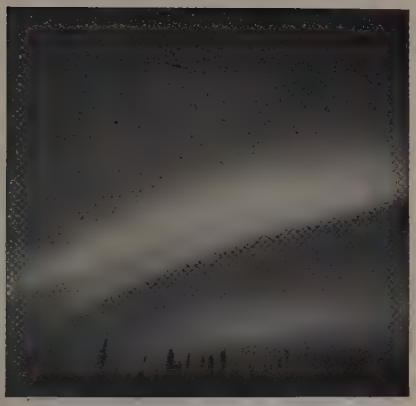


O 40

Typical simultaneous photographs of aurora taken at Coral Rapids (C) and Onakawana (O): No. 34 taken Jan. 26, 1931; No. 39 and 40 taken Jan. 27, 1931.



C 41



O 41



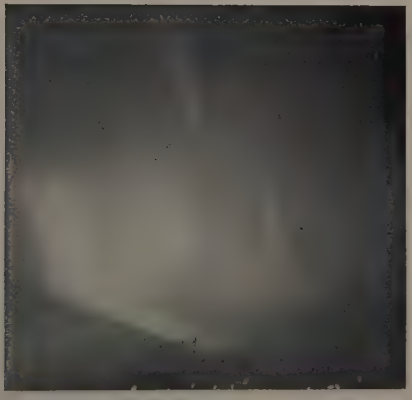
C 45



O 45



C 48



O 48

Typical simultaneous photographs of aurora taken at Coral Rapids (C) and Onakawana (O): No. 41 taken Jan. 27, 1931; No. 45 and 48 taken Feb. 13, 1931.

ON THE SOUND FIELD IN THE NEIGHBORHOOD OF AN OSCILLATING PLANE DISK[1]

By R. Rüedy[2]

Abstract

With the aid of recent theories (H. Stenzel, N. W. McLachlan, H. Backhaus) the velocity potential and pressure distribution at points in the field of vibrating solid disks of 1 to 20 cm. diameter are calculated for a number of frequencies of practical importance. The graphs drawn from these values apply also to very high frequencies, but smaller disks (1 to 20 mm.). They illustrate the gradual transition from spherical distribution to directed transmission.

The calculation of the sound field in the neighborhood of solid disks or pistons set into an opening of a practically infinite wall and vibrating to and fro with a maximum velocity, v_0, has become of great interest for a number of applications. The methods hitherto used were borrowed from optical theories (4, 5).

At points in the neighborhood of the axis the amplitude distribution may be found by dividing the disk into a number of half-wave zones, and at large distances from the centre the angle with the normal for the first minimum of intensity is equal to $\sin^{-1} 0.61 \frac{\lambda}{\rho}$. When the diameter of the piston surface is small compared with the wave-length, then the motion is distributed spherically with the disk as centre. But when the wave-length is much smaller than the diameter, zones of maximum and minimum intensity are produced along certain directions around the normal to the centre of the piston, and also along certain more or less circular zones. Close to the vibrating body the wave must be nearly plane.

This theory is only approximate, and whereas in the optical case the number only and the position of the main beams are of most interest, in the sound field the shape of the different beams matters, and effects close to the source have to be studied. A more rigorous treatment has been given recently by H. Backhaus (1).

Taking Lord Rayleigh's equation for the velocity potential, φ, in the case of the circular piston of radius ρ,

$$\varphi = -\frac{1}{2\pi} \iint \frac{\partial \varphi}{\partial n} \frac{e^{-ikr}}{r} \, dS,$$

or for a solid body,

$$\varphi = -\frac{1}{2\pi} \frac{\partial \varphi}{\partial n} \iint \frac{e^{-ikr}}{r} \, dS.$$

From the velocity potential the pressure changes at any moment are

$$p = -s \frac{\partial \varphi}{\partial t},$$

[1] *Manuscript received June 18, 1931.*
Contribution from the National Research Laboratories, Ottawa. This paper was read before Section III of the Royal Society of Canada in May, 1931.

[2] *Junior Research Investigator, National Research Laboratories, Ottawa.*

and the velocity along the direction to the centre of the disk

$$v = \frac{\partial \varphi}{\partial r}.$$

Assuming that the integral on which φ depends may be expressed as a sum of spherical harmonics, the new theory gives for the frequency, f, at a point r, θ.

$$\varphi = -v_0 \rho \sqrt{\frac{\pi}{2}} \sqrt{\frac{B_1^2 + B_2^2}{kr}} \sin(2\pi ft + \psi),$$

$$\tan \psi = \frac{B_2}{B_1},$$

with

$$k = \frac{2\pi}{\lambda}$$

$$B_1 = A_0(k\rho) P_0(\cos\theta) J_{\frac{1}{2}}(kr) + A_2(k\rho) P_2(\cos\theta) J_{\frac{3}{2}}(kr) + A_4(k\rho) P_4(\cos\theta) J_{\frac{5}{2}}(kr) + \ldots$$

$$B_2 = A_0(k\rho) P_0(\cos\theta) J_{-\frac{1}{2}}(kr) + A_2(k\rho) P_2(\cos\theta) J_{-\frac{3}{2}}(kr) + A_4(k\rho) P_4(\cos\theta) J_{-\frac{5}{2}}(kr) + \ldots$$

$$A_{2n}(k\rho) = (-1)^n (4n+1) \left[\frac{1}{k\rho} - \frac{1}{2n} \sum (-1)^{n-m} \frac{1.3.5 \ldots (2n+2m-1)}{m!(n-m)!} \frac{\sqrt{\frac{\pi}{2}}\, J_{m-\frac{1}{2}}(k\rho)}{(k\rho)^{m+\frac{1}{2}}} \right]$$

for instance,

$$\frac{8}{9} k\rho\, A_4(k\rho) = 8 - \cos k\rho \left(3 - \frac{105}{(k\rho)^2}\right) + \frac{\sin k\rho}{k\rho}\left(30 - \frac{105}{(k\rho)^2}\right)$$

$$-\frac{16}{13} k\rho\, A_6(k\rho) = 16 + \cos k\rho \left(5 - \frac{945}{(k\rho)^2} + \frac{10395}{(k\rho)^4}\right) - \frac{\sin k\rho}{k\rho}\left(105 - \frac{4410}{(k\rho)^2} + \frac{10395}{(k\rho)^4}\right)$$

$$\frac{16}{17} k\rho\, A_8(k\rho) = 16 - \cos k\rho \left(\frac{35}{8} - \frac{10395}{4(k\rho)^2} + \frac{1216215}{8(k\rho)^4} - \frac{10135125}{8(k\rho)^6}\right)$$

$$+ \frac{\sin k\rho}{k\rho}\left(\frac{315}{2} - \frac{100485}{4(k\rho)^2} + \frac{4590590}{8(k\rho)^4} - \frac{10135125}{8(k\rho)^6}\right).$$

P = spherical harmonics; J = Bessel functions.

This solution is valid for points of the sound field facing the piston lying outside a hemispherical cap of radius equal to that of the disk; it also applies to all the points lying upon the normal to the centre of the disk. In comparison with the approximate theory the rigorous treatment gives a smaller number of directions and points in which there is no pressure change, less narrow and stronger sidebeams and therefore more overlapping. This feature has been emphasized in experiments on ultrasonics (2) and has also been verified in the case of low frequency waves.

In an attempt to extend the solution to points lying inside the hemispherical cap, at least for ultrasonic frequencies, calculations were made for the frequency, 54100 cycles per sec., or a wave-length of 0.63 cm. as emitted by pistons of diameters $2\rho = 0.2$, 0.63, 1.26 and 1.88 cm. When multiplied by ten the results are also valid for wave-lengths ten times longer, provided the piston diameters and distances are enlarged in the same ratio so that the values of $\frac{2\pi\rho}{\lambda}$ and $\frac{2\pi r}{\lambda}$ remain unchanged, that is, for a frequency of 5410 periods per sec. and piston diameters of 2, 6.3, 12.6 and 18.8 cm., or loud-speaker dimensions.

To avoid too complicated figures, only the curves $\sqrt{\frac{B_1^2+B_2^2}{kr}}$ for angles θ equal to zero, thirty or sixty degrees from the normal, have been plotted. The curves show the gradual transition from diffuse spherical to directed radiation affected by interference, and as their accuracy may be tested experimentally, they are thought to be of general interest as interference effects on a convenient scale.

As for sound waves the pressure amplitude of the air is proportional to the velocity potential φ, the curves constructed from the computed values may be considered as giving the maximum pressure amplitudes occurring at any time at points before the disk. In order to obtain the instantaneous distribution

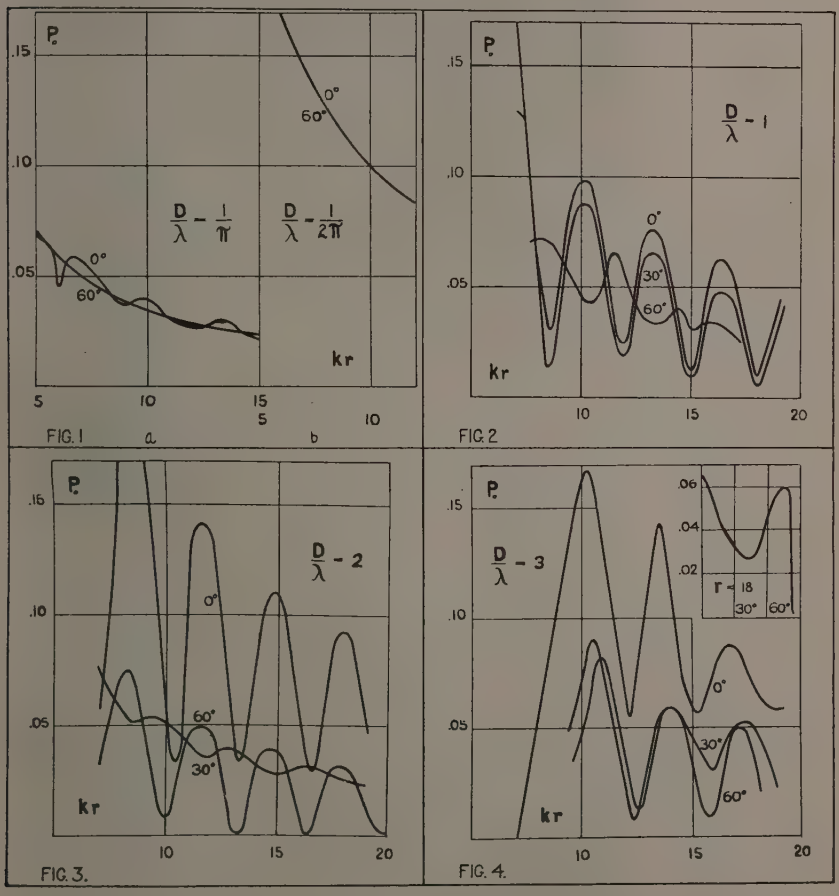


FIG. 1–4. *Pressure amplitudes in the sound field of a vibrating solid disk of diameter D.*

of pressure the phase angle, ψ, must be taken into account; the slope of the tangents to the curves gives then the velocity of vibration in the direction to the centre of the disk.

With regard to the absolute values of the pressure changes at any point (c = velocity of sound),

$$p = -2\pi s v_0 \rho f \sqrt{\frac{\pi}{2}} \sqrt{\frac{B_1^2 + B_2^2}{kr}} \cos(2\pi f t + \psi),$$

or

$$p = -1.253\, c v_0 (k\rho) \sqrt{\frac{B_1^2 + B_2^2}{kr}} \cos(2\pi f t + \psi).$$

When plotting the values of the square root only as has been done in drawing the graphs, the absolute value of the amplitude at a point may be found by multiplying by $1.253\, scv_0\, k\rho$ (where s is the density of the air, c the velocity of sound), except in the case of Fig. 1*b* where the factor is $0.132\, scv_0$; actually there are phase differences between the different values. The points have been computed for every one-half centimetre distance, putting $k = 1$.

In the first case, diameter equal to 1 mm., the curves for all the angles from 0° to 90° from the normal practically coincide and show an almost perfectly regular decrease for increasing distances. The sound waves are essentially spherical waves.

In the case where the diameter is equal to 2 mm., marked interference effects make themselves felt; at certain distances the amplitudes remain small, but there is little difference as to maximum amplitudes when different angles are considered.

In the second figure, diameter comparable to wave-length, $k\rho = \pi$, the interference effects cut deeper; at certain distances the amplitudes tend to zero, thus giving the appearance of node formation. At the same time, the distribution valid for zero degree differs completely from that at ninety degrees and the tendency to form side beams becomes apparent.

For $k\rho = 3\pi$(in Fig. 4) one point upon the normal to the disk may be found in which the pressure amplitude is zero. Several attempts to verify this feature have failed. The number of points on the normal with no pressure change would increase when $k\rho$ increases. It is possible that frictional forces tend to produce a flow of the air and smooth out the differences between the points of greatest and least changes.

The up-and-down fluctuations of the pressure amplitude along the different directions grow gradually smaller as larger values of r are examined, and finally at distances at which the lines connecting a point with all the different surface elements of the disk may be considered as parallel lines, Airy's solution holds(6).

$$\varphi = -\frac{\rho^2 v_0}{r} \frac{J_1(k\rho \sin\theta)}{k\rho \sin\theta} \cos(2\pi f t - kr),$$

$$p = -\frac{s\partial\varphi}{\partial t} = s c v_0 k\rho \frac{J_1(k\rho \sin\theta)}{kr \sin\theta} \sin(2\pi f t - kr).$$

The graphs may also be used in another way. When the curves for $k\rho = 3\pi$ are taken as applying to a solid vibrating disk of diameter 6π cm., they represent the pressure distribution for a wave of 5410 cycles (or a wave-length of 6.28 cm. in air), that is, for a very high note. The graphs for the case $k\rho = 2\pi$ may then be considered as applying to the same disk but to a three times longer wave-length and at three times longer distances; those for $k\rho = \pi$ to six times enlarged dimensions (frequency 900 cycles), and for $k\rho = 1$ the dimensions would be measured in dm. instead of in cm. For this latter case and all the lower frequencies (<500 cycles), the distribution is nearly hemispherical, and the formula contains only terms with A_0 so that (Fig. 1-*b*.)

$$p = -s\,c\,v_0 \frac{1-\cos k\rho}{kr} \cos(2\pi ft+\psi),$$

because

$$\frac{2}{\pi y} = J^2_{\frac{1}{2}}(y)+J^2_{-\frac{1}{2}}(y).$$

The different curves thus show the main features of the sound field for the frequencies of practical importance and the deviation from uniform distribution when high frequencies are considered. It is important that as far as measurements and practical uses are concerned the ideal disk oscillating at high frequency may be closely imitated if instead of cutting thin quartz slabs as circular disks, the different diameters are made proportional to the square root of the modulus of elasticity in that direction (7). At lower frequencies the disks may be driven electromagnetically. As the conditions in the sound field may be determined by merely measuring the amplitude of vibration of the disk, the disks are suitable for standard and precision sound sources. The theory applies to more complicated structures provided they can be resolved into or replaced by a number of circular disks.

It is desirable to extend the graphs to larger values of $k\rho$; the amount of computation increases rapidly however with $k\rho$ and suitable tables for the higher order Bessel functions will have to be prepared.

Acknowledgment

This work was proposed by Dr. R. W. Boyle and thanks are due to him for suggestions received.

References

1. Backhaus, H. Ann. Physik, 5: 1-35. 1930.
2. Boyle, R. W., Lehmann, T. F. and Reid, C. D. Trans. Roy. Soc. Can. 19 III: 167-196. 1925.
3. Lindsay, R. B. Phys. Rev. 32: 515-519. 1928.
4. McLachlan, N. W. Proc. Roy. Soc. Lond. 122A: 604-609. 1929.
5. Stenzel, H. Elek. Nachr.-Tech. 4: 239-251. 1927.
6. Stenzel, H. Ann. Physik, 7: 947-982. 1930.
7. Straubel, H. Physik. Z. 32: 222. 1931.

STUDIES ON LIGNIN AND RELATED COMPOUNDS
IX. ETHERS OF GLYCOL-LIGNIN[1]

By Harold Hibbert[2] and Léo Marion[3]

Abstract

Glycol-lignin prepared from spruce wood has been found to form a sodium salt which reacts with chloro-derivatives, giving rise to the corresponding glycol-lignin ethers. These compounds are insoluble in aqueous sodium hydroxide but still contain free hydroxyl groups which can be methylated without the ethereal linkage being broken. Hence glycol-lignin contains several free hydroxyl groups, one or more of which possess acidic properties. Both the methoxymethyl ether and the 2:4-dinitrophenyl ether of glycol-lignin have been prepared. The evidence would seem to indicate the presence of both phenolic and aliphatic hydroxyl groups.

Introduction

Recently an account was given of some investigations (5, 6) which supplied evidence that could be interpreted as an indication of the presence in glycol-lignin of a phenolic nucleus. The work described in the present paper and carried out simultaneously with the latter, was designed to emphasize any difference in the nature of the hydroxyl groups present in glycol-lignin.

Glycol-lignin, isolated from spruce wood by means of glycol containing a trace of hydrogen chloride, contains free hydroxyl groups (4). It appeared possible, therefore, to prepare a sodium salt which might be made to react with various chloro-derivatives to yield the corresponding ethers. The present work is concerned with the preparation of such ethers of glycol-lignin and a study of their properties.

Hoering and Baum (7) prepared methoxymethyl phenyl ether by allowing sodium phenolate to react with chlorodimethyl ether. By the application of the same method, Pauly and Wäscher (9) and, later, Pauly and Feuerstein (8) succeeded in obtaining coniferyl aldehyde from vanillin. In a somewhat similar manner, Freudenberg and Hess (1) prepared a *p*-toluene sulphonic ester of Willstätter lignin which they were able to isolate and decompose by treatment with hydroxylamine. Their purpose was to determine the nature of the hydroxyl group with which the sulphonic radical was combined. Assuming a molecular weight for lignin of the order of 800, they concluded that it contained four aliphatic, or hydroaromatic, hydroxyl groups, and one phenolic group. Using a lignin prepared by a different method, however, Freudenberg, Zocher and Dürr (2) later concluded that phenolic groups are absent in the lignin complex.

As a first step towards the synthesis of ethers of glycol-lignin, the sodium salt of the latter was prepared from its alcoholic solution by the addition of sodium

[1] *Manuscript received June 24, 1931.*
Contribution from the Department of Industrial and Cellulose Chemistry, McGill University, Montreal, Canada, with financial assistance from the National Research Council of Canada and the Canadian Pulp and Paper Association.

[2] *Professor of Industrial and Cellulose Chemistry, McGill University.*

[3] *Assistant Research Chemist, National Research Laboratories, Ottawa, and holder, at the time, of a studentship under the National Research Council.*

ethylate. The salt is a very hygroscopic, light-colored, amorphous powder which is oxidized to a dark-brown mass when left exposed to the air, a property characteristic of the sodium salts of polyhydroxy phenols.

The sodium salt of glycol-lignin, when suspended in toluene and allowed to react under suitable conditions with 1:2:4 chlorodinitrobenzene, yielded a lemon-yellow compound containing 2.74% nitrogen and having a methoxyl content of 13.60%. Its molecular weight, determined cryoscopically in glacial acetic acid, had an average value of 499. The ether is insoluble in dilute sodium hydroxide, but undergoes methylation by the agency of dimethyl sulphate and potassium hydroxide, the methoxyl content being increased to 17.0% (average of four determinations). If, however, the temperature during methylation be allowed to rise to 90-100° C., and the reaction mixture to become alkaline, the ether group is removed and the insoluble lignin derivative has, after purification, a methoxyl content of 22.63%. Fully methylated glycol-lignin has a methoxyl value of 26.26%. If, therefore, it be assumed that the difference between the two compounds consists in one acidic hydroxyl group being free in the one case and methylated in the other, the molecular weight of fully methylated lignin is found by calculation to be 766. This figure, however, can only be considered as representing the order of the size of the molecule, owing to the possibility of the product left after removal of the dinitrophenyl group having undergone partial methylation. In fact, the molecular weight of fully methylated glycol-lignin can be calculated from the methoxyl values of fully methylated glycol-lignin (26.26%) and of the fully methylated ether (17%) to be 615.

The results show, however, that glycol-lignin contains at least two types of hydroxyl groups and that those responsible for the solubility of glycol-lignin in dilute sodium hydroxide can combine with the sodium present in sodium ethoxide to form a well-defined salt. Fuchs and Horn (3) found that lignin isolated from spruce wood contains two kinds of hydroxyl groups, some undergoing methylation through the agency of diazomethane while the others are inert towards this reagent; this is in harmony with the above conclusion.

Chlorodimethyl ether also reacts with the sodium salt of glycol-lignin, yielding the methoxymethyl ether of glycol-lignin. The methoxyl content of the compound has an average value of 18.9%. As in the case of the dinitrophenyl ether this compound is insoluble in dilute sodium hydroxide but can be methylated.

Experimental

Preparation of the Sodium Salt of Glycol-lignin

About 2 gm. of moist glycol-lignin* was dissolved in 100 cc. of absolute alcohol and the solution cooled to −10° C. A solution of sodium ethylate (1 gm. metallic sodium in 20 cc. absolute alcohol), previously cooled to the same temperature, was gradually added, with occasional shaking, to the cooled alcoholic solution. The sodium salt of glycol-lignin immediately separated as

**Glycol-lignin was prepared and purified as described in a previous communication (4) and kept moist in a well-stoppered brown flask until needed.*

a light-colored precipitate which was washed by decantation with cold absolute alcohol, filtered as rapidly as possible, and washed on the filter, first with absolute alcohol and then with anhydrous ether. It was spread on a watch glass and dried over phosphorus pentoxide in an evacuated desiccator. The product was left as a hygroscopic powder which gradually turned dark brown when left exposed to the air. It was insoluble in ether and alcohol (absolute); soluble in water; also soluble in pyridine with decomposition. The substance precipitated from the pyridine solution by the addition of ether or absolute alcohol is no longer soluble in water.

Dinitrophenyl Ether of Glycol-lignin

The dry sodium salt of glycol-lignin (2.5 gm.) was suspended in 50 cc. of toluene in a flask, equipped with a mechanical stirrer, and heated in a bath kept at 70° C. To this was then added a solution of 2 gm. of 1:2:4 chlorodinitrobenzene in 50 cc. of toluene, and the mixture stirred at 70° C. The dinitrophenyl ether gradually separated as a lemon-yellow precipitate, together with sodium chloride. After five hours the mixture was cooled and water added to dissolve the sodium chloride and any unchanged sodium salt of lignin. The solid ether was then filtered, washed with water, and dried under reduced pressure. It was purified by dissolving in glacial acetic acid (about 40 cc.) and reprecipitated by pouring the solution into 500 cc. water. The ether thus obtained, after filtering, washing and drying (yield 2.6 gm.) was a light lemon-yellow, amorphous powder soluble in glacial acetic acid, very slightly soluble in dilute alcohol and insoluble in toluene, acetone, chloroform and ethyl acetate. Analysis: C, 57.82, 57.70%; H, 5.93, 5.73%; N, 2.71, 2.78%; (OCH_3), 13.84, 13.60, 13.26%. Mol. wt. in glacial acetic acid: sample, 0.0498, 0.0604 gm., Δ,0.193, 0.231° C., mol. wt., 496, 502.

Methylation of Glycol-lignin Dinitrophenyl Ether

The dinitrophenyl ether (0.5 gm.) was suspended in 5 cc. of 1% sodium hydroxide and methylated by the addition of 5 cc. of dimethyl sulphate and 5 cc. of 50% potassium hydroxide at 43-45° C. Both reagents were added at such a rate as to maintain the mixture as nearly neutral as possible throughout the addition. The reaction product was filtered, washed and then subjected to a second similar methylation. Following this second treatment, the product was filtered, washed with water and dried under reduced pressure over phosphorus pentoxide. It was obtained as a light-brown powder, insoluble in sodium hydroxide, alcohol or glacial acetic acid. Analysis: CH_3O, 17.15, 16.42, 16.93, 17.55%. If during methylation, the temperature was allowed to rise to 90-100° C. and the reaction mixture to become alkaline, the dinitrophenyl ether group was removed, leaving a partially methylated lignin derivative. Analysis: CH_3O, 22.78, 22.48%.

Methoxymethyl Ether of Glycol-lignin

This ether was obtained by the interaction of chlorodimethyl ether with the sodium salt of glycol-lignin. Two grams of the salt was suspended in pure, dry toluene, and the mixture, after cooling in ice, stirred with 15 cc. of chloro-

dimethyl ether for one hour. The temperature of the reaction mixture was then allowed to rise to room temperature and the product poured into a large volume of water to dissolve any of the unchanged sodium salt of lignin and the sodium chloride formed. The methoxymethyl ether, after filtering, washing and drying under reduced pressure over phosphorus pentoxide, was a light-brown, amorphous powder, insoluble in toluene, ether and dilute caustic soda. Analysis: C, 61.79, 61.42%; H, 6.32, 6.06%; CH_3O, 19.08, 19.61%.

References

1. Freudenberg, K. and Hess, H. Ann. 448: 121-133. 1926.
2. Freudenberg, K., Zocher, H. and Dürr, W. Ber. 62: 1814-1823. 1929.
3. Fuchs, W. and Horn, O. Ber. 62: 1691-1693. 1929.
4. Hibbert, H. and Marion, L. Can. J. Research, 2: 364-375. 1930.
5. Hibbert, H. and Sankey, C. A. Can. J. Research, 4:110-118. 1931.
6. Hibbert, H. and Taylor, K. A. Can. J. Research, 4: 240-253. 1931.
7. Hoering, P. and Baum, F. Centr. 1: 1680. 1909.
8. Pauly, H. and Feuerstein, K. Ber. 62: 297-311. 1929.
9. Pauly, H. and Wäscher, K. Ber. 56: 603-610. 1923.

THE PREPARATION AND PHYSICAL PROPERTIES OF ETHYL AND METHYL ACETYLENE[1]

By F. R. Morehouse[2] and O. Maass[3]

Abstract

The method for preparing an alkyl acetylene by the action of an alkyl halide on sodium dissolved in liquid ammonia was modified in certain details. Ethyl and methyl acetylene were prepared and purified and their melting and boiling points determined. Vapor pressures, densities and surface tensions were measured over a considerable temperature range. These properties together with constants calculated from the data are compared with similar data for paraffins and olefines.

A systematic study of the physical properties of aliphatic hydrocarbons has been undertaken in this laboratory. Those containing two and three carbon atoms were investigated by Wright and Maass (4) and the properties of the four-carbon paraffins and olefines were determined by Coffin and Maass (1). In this paper the first of the four-carbon acetylenes is the subject of investigation.

Certain details in the method of preparation which lead to better yields are described. The method of purification was similar to that used by Coffin and Maass (1). Since this is a marked improvement over that used by Wright and Maass (4), where small amounts of material are concerned, the preparation of methyl acetylene also was undertaken to see if, when purified in this way, the results of Wright and Maass would be confirmed or not.

Preparation

The acetylene compounds were prepared by the action of the alkyl halides on sodium acetylide in a manner analogous to that described by Lebeau and Picon (2). At first the type of apparatus described by Russell and Maass (3) was employed and special care was taken to dry the ammonia in sodium hydroxide towers and to add the alkyl halide after the excess acetylene had been removed. It was found that a very poor yield was obtained even though, after addition of alkyl halide, the reaction mixture had been allowed to stand for some time. This was attributed to the low temperature ($-78°$ C.) of the carbon dioxide-ether bath in which it was kept. The maximum temperature at which the reaction mixture can be maintained is of course governed by the boiling point of the ammonia.

The apparatus was therefore modified by the addition of a reflux condenser (Fig. 2) whereby the alkyl halide could be added while the mixture was being refluxed; *i.e.*, at the highest possible temperature. Fig. 1 represents the apparatus used. It was constructed so as to be adapted to the preparation of other acetylenic compounds and to be a permanent set-up.

[1] *Manuscript received March 16, 1931.*
Contribution from the Physical Chemistry Laboratory, McGill University, Montreal, Canada.

[2] *Holder, at the time, of a studentship under the National Research Council of Canada.*
[3] *Professor of Physical Chemistry, McGill University.*

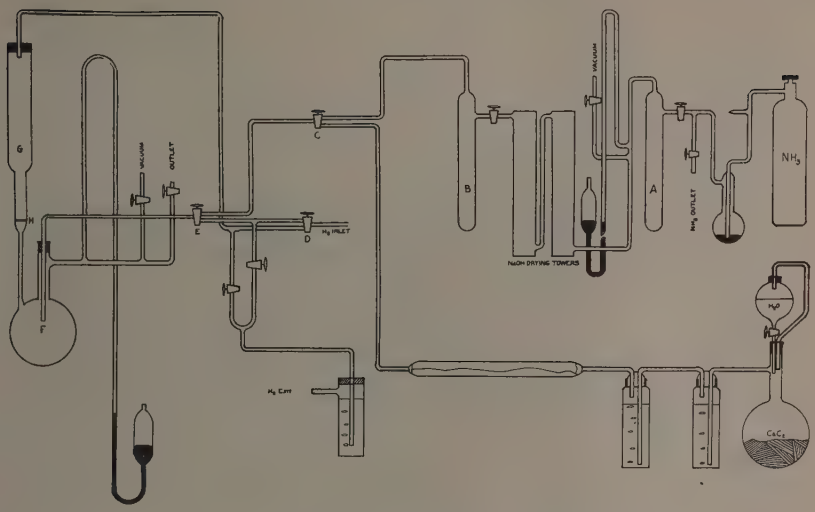


FIG. 1. *Apparatus used in preparation of alkyl acetylenes.*

The ammonia is passed through sodium hydroxide drying towers and then condensed in the reaction flask, *F*, where it dissolves the sodium, previously melted in an atmosphere of hydrogen in the tube *G*. It is then strained, while molten, through the perforated porous plate, *H*, in the reaction flask. When the sodium has been added tube *G* is sealed off. Pure acetylene is then passed through the solution of sodium in liquid ammonia until the blue color disappears after which the reflux condenser, Fig. 2, is attached to the reaction flask and the contents allowed to reflux for some time to remove any dissolved acetylene. The reflux condenser consists of a tube jacketed with carbon dioxide-ether mixture. A dropping funnel is attached for the addition of the alkyl halide. After it has been added the contents of the flask are allowed to boil off through a scrubbing apparatus (Fig. 3) in which the flask, *C*, contains a small amount of water kept acid by the gradual addition of sulphuric acid from a dropping funnel, a piece of litmus paper in the solution acting as indicator. In this way complete removal of ammonia was assured. Yields obtained by the use of this type of separator were far greater than when the larger part of the ammonia was first removed by passing the gases through a large quantity of water; 70% yields were obtained.

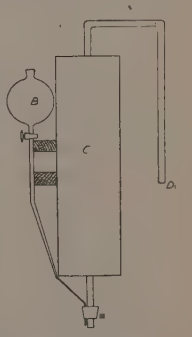


FIG. 2. *Reflux condenser.*

The alkyl acetylene compound was then collected and purified by the system of fractional distillation used by Coffin and Maass. Vapor density determinations were made at different pressures on the ethyl acetylene, giving a result which corresponded to the theoretical molecular weight. Pure methyl

acetylene was obtained by distilling until a constant vapor pressure for different fractions was obtained. The vapor pressures of the final two fractions were 54.5 and 54.3 cm. Hg. at −30° C.

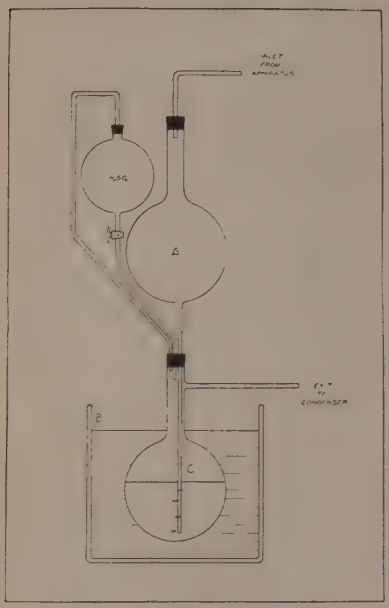


FIG. 3. *Scrubbing apparatus.*

Ethyl and methyl acetylene were prepared twice with slight changes in the method of procedure. In the first preparation of ethyl acetylene it was never at any time in contact with an acid separator which might have polymerized it. In the second preparation, where better yields were obtained by the use of the acid separator described above, the alkyl acetylene was in contact with acid both in the separator and also during passage over phosphorus pentoxide for removal of small traces of water. Most of the water was first removed by chilling and then filtering off the ethyl acetylene. Although propylene polymerizes (4) on being passed over phosphorus pentoxide, ethyl acetylene does not appear to do so since the vapor pressures and boiling points were found to be the same whether it did or did not come into contact with an acid medium.

The useful information was therefore obtained that no secondary products are formed by passing the alkyl acetylene through dilute sulphuric acid or (after removal of most of the water) by passing it over phosphorus pentoxide. Consequently both ammonia and water can be completely removed before fractionation. The data for ethyl acetylene given below are for two samples prepared separately from the start. The results obtained with preparation I are from the sample which had never been in contact with an acid solution or phosphorus pentoxide, while those for preparation II are from the sample which was passed through the acid solution and over phosphorus pentoxide. Both sets of data are seen to agree.

Determination of the Melting Point

Melting points were determined by freezing the hydrocarbons in small bulbs which were immersed in a bath of gasoline contained in a transparent Dewar flask. A Pyrex test tube was held in the bath and liquid air was put in it until the desired temperatures were reached. Stirring of the gasoline was effected by a current of dry air.

Temperatures were measured by a standardized thermometer completely immersed in the liquid. The melting points of ethyl and methyl acetylene were found to be −122.5° and −101.5° C. respectively.

Boiling Point

Ethyl Acetylene

The boiling point of ethyl acetylene has been found by different investigators and considerable disagreement occurs in the literature. The boiling point was found from the vapor pressure curve taken over a suitable temperature range. Temperatures were maintained by an air-stirred ether bath cooled with solid carbon dioxide and measured with a standard thermometer. The following tables give the vapor pressures for preparation I and preparation II from which the boiling point in both sets of data is found to be +8° C.

TABLE I

VAPOR PRESSURES OF ETHYL ACETYLENE

Preparation I				Preparation II			
Temp., ° C.	Vapor pressure, cm. Hg.	Temp., ° C.	Vapor pressure, cm. Hg.	Temp., ° C.	Vapor pressure, cm. Hg.	Temp., ° C.	Vapor pressure, cm. Hg.
−38.8	6.80	−5.7	43.10	−32.4	11.46	−7.0	40.85
−30.5	12.38	−1.3	50.78	−24.1	17.93	−4.5	46.03
−21.3	20.67	+3.5	61.96	−21.3	19.93	+8.0	74.42
−16.9	26.18	+9.1	77.66	−17.4	24.81	+9.5	78.80
−10.8	34.60			−11.4	33.57		

Methyl Acetylene

The boiling point of methyl acetylene was found in the same way. The boiling point in preparation I (−23.3° C.) was found to be the same as that in preparation II within 0.1° C. This value for the boiling point shows fair agreement with that (−23.0° C.) obtained by R. N. Meinert and C. D. Hurd (5).

TABLE II

VAPOR PRESSURES OF METHYL ACETYLENE

Preparation I				Preparation II			
Temp., ° C.	Vapor pressure, cm. Hg.	Temp., ° C.	Vapor pressure, cm. Hg.	Temp., ° C.	Vapor pressure, cm. Hg.	Temp., ° C.	Vapor pressure, cm. Hg.
−54.1	15.88	−33.4	48.74	−50.1	20.03	−25.2	71.15
−49.6	20.46	−28.5	60.80	−39.6	35.18	−22.6	80.01
−44.0	27.64	−25.5	67.80	−30.2	55.90	−21.1	83.80
−36.5	42.17	−22.5	79.70				

Density

Density determinations were made by the dilatometer method described by Wright and Maass (4). The dilatometer was glass-sealed to the apparatus and filled by distillation. In the tables below are given the densities of ethyl and methyl acetylene for preparations I and II.

TABLE III

DENSITIES OF ETHYL ACETYLENE

Preparation I		Preparation II	
Temp., ° C.	Density	Temp., ° C.	Density
−28.0	0.7095	−31.3	0.7119
−20.0	0.6997	−23.0	0.7028
−15.0	0.6949	−11.0	0.6899
−10.5	0.6900	− 0.2	0.6784
− 5.0	0.6836	+ 8.9	0.6685
− 0.3	0.6784		
+ 5.6	0.6706		

TABLE IV

DENSITIES OF METHYL ACETYLENE

Preparation I				Preparation II			
Temp.,°C.	Density	Temp.,°C.	Density	Temp.,°C.	Density	Temp.,°C.	Density
−14.4	0.6617	−36.8	0.6873	− 0.5	0.6447	−33.0	0.6814
−17.5	0.6652	−42.4	0.6936	−12.7	0.6582	−40.9	0.6911
−22.7	0.6710	−47.4	0.6992	−24.2	0.6711	−51.9	0.7039
−26.5	0.6759	−53.1	0.7065				
−32.5	0.6827	−57.0	0.7109				

Surface Tension

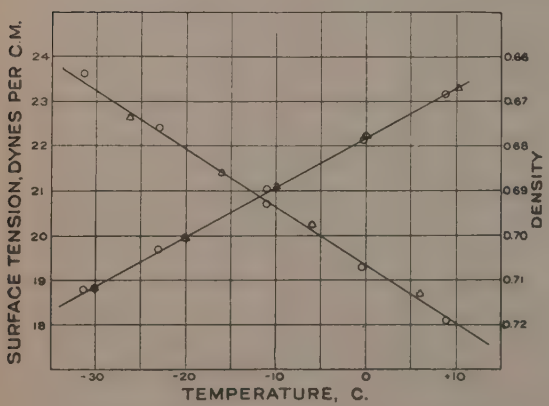


FIG. 4. *Surface tension and density of ethyl acetylene.*

The surface tension of ethyl acetylene was found by the use of a capillarimeter which was glass-sealed to the purifying apparatus and filled by distillation. A description of this method and the accuracy obtained is to be found in the paper by Wright and Maass (4) referred to above. The following table gives the values obtained for surface tension. The curves for surface tension derived from both sets of data for ethyl acetylene are shown in Fig. 4.

TABLE V

SURFACE TENSION OF METHYL AND ETHYL ACETYLENE

Methyl acetylene*		Ethyl acetylene			
Preparation II		Preparation I		Preparation II	
Temp., °C.	Surface tension	Temp., ° C.	Surface tension	Temp., °C.	Surface tension
−11.5	18.91	−26.3	22.65	−31.3	23.62
−21.0	20.37	−16.0	21.40	−23.0	22.44
−31.6	22.00	− 6.3	20.25	−11.0	20.77
−38.4	23.08	+ 6.5	18.70	− 0.2	19.32
				+ 8.9	18.13

**The surface tension of methyl acetylene was found only under those conditions pertaining to preparation II.*

Discussion

Table VI is a compilation of the data obtained for the hydrocarbons of the acetylene series. A table containing similar data for the paraffins and olefines can be found in a paper by Coffin and Maass (1).

TABLE VI

PHYSICAL PROPERTIES OF ACETYLENES

Property	Acetylene	Allylene	Ethyl acetylene	Property	Acetylene	Allylene	Ethyl acetylene
Melting point, ° C.	−81	−101.5	−122.5	Critical temperature, °C.	39.7	121.6	190.5
Boiling point, ° C.	−83.6	−23.3	+8.6	Molecular volume at b.p., °C.	41.9	59.7	80.8
Molecular latent heat of evaporation, cal.	4273	5562	5983	Parachor: Observed	88.1	127.5	167.2
Surface tension at b.p. dynes/cm.	19.6	20.7	18.3	Calculated	90.4	129.4	168.6
Total surface energy	56.0	59.42	53.5	Difference as % of observed value	2.6	1.5	0.8
Ramsay and Shield's constant	2.09	1.99	1.96				
Trouton's constant	—	22.3	21.3				

The new data obtained from allylene do not differ markedly from that obtained by Maass and Wright (4) except in so far as the boiling point is concerned. This was found to be considerably higher. The amount of material used by Maass and Wright was considerably less, and it is possible that in the determination of the vapor pressures all the air above the liquid had not been removed. In any case, because of the larger amount of material available for purification, the data for allylene given above may be considered more accurate.

The physical constants obtained for ethyl acetylene fall in line with the analogous constants obtained for the olefines and paraffins on one hand and the two lower acetylenes on the other. The values obtained for Trouton's constant and for Ramsay and Shield's constant show that ethyl acetylene may

be regarded as unassociated liquid just as all the other hydrocarbons which have been examined. As a member of the acetylenes, ethyl acetylene shows a greater force of molecular attraction than the olefines and paraffins. The latent heat of evaporation, the total surface energy, the boiling point and the critical temperature are all considerably higher than the similar constants for the butanes and butylenes. The melting point is higher than for any other "four-carbon" hydrocarbon, and that it is the lowest as compared to the two lower acetylenes is to be expected. There seems to be no question but that the acetylenes have what some like to call a larger residual valency than the corresponding olefines and paraffins.

The molecular volume at the boiling point of acetylene, allylene and ethyl acetylene can only be brought into line with Kopp's values and not those of Le Bas; ethyl acetylene showing the increase of 3.8 units for triple bond on saturation which Kopp found. The two lower members of the acetylene series show the increase above this value which is to be expected from their position in the acetylene series.

The values for the observed parachor are given in Table VI for acetylene, allylene and ethyl acetylene, and there is agreement between these values and those calculated on the basis of Sugden's values. That ethyl acetylene shows the best agreement of the three is to be expected, since it is further up in the series where greater uniformity is to be expected.

References

1. Coffin, C. C. and Maass, O. J. Am. Chem. Soc. 50: 1427-1437. 1928.
2. Lebeau, P. and Picon, M. Compt. rend. 156: 1077-1079. 1913.
3. Maass, O. and Russell, J. J. Am. Chem. Soc. 43: 1227-1230. 1921.
4. Maass, O. and Wright, C. H. J. Am. Chem. Soc. 43: 1098-1111. 1921.
5. Meinert, R. N. and Hurd, C. D. J. Am. Chem. Soc. 52: 4540-4549. 1930.

FACTORS WHICH INFLUENCE SPONTANEOUS SELF-FERTILIZATION IN SWEET CLOVER (MELILOTUS)[1]

By L. E. Kirk[2] and T. M. Stevenson[3]

Abstract

Seven factors have been described which seem to influence the tendency of sweet clover plants to produce seed by spontaneous self-fertilization. These are: length of stamens; stage of flower development when pollen is liberated from the pollen sacs; distribution of free pollen within the flower; size of cavity in the upper part of the keel; amount of pollen; condition of the pollen; and receptivity of the stigma.

Spontaneous self-fertilization is the normal condition in certain plants of white blossom sweet clover, *i.e.*, *M. alba*. This was not found to occur in any variety of yellow blossom sweet clover with the exception of "Redfield Yellow". True breeding lines of *M. alba* have been isolated consisting of naturally self-fertilized plants and other lines also which will not produce seed unless the flowers are manipulated. *M. alba* consists of a mixture of normally self-fertilized plants and plants that are random pollinated. This is especially true of certain varieties such as "Arctic". In some plants of *M. alba* an abundance of germinating pollen grains were found adhering to the stigmas before the flowers opened, thus precluding the possibility of natural crossing. Practically 100% of the flowers on such a plant produced selfed seed. The character of spontaneous self-fertilization exhibited clear cut segregation in a selfed line of *M. alba*. The importance of this character in relation to breeding improved varieties and pure seed production is emphasized.

The efficiency of the "suction" method of emasculating sweet clover flowers depends on the peculiarities of the latter. This method is quite reliable with many plants but very unsatisfactory with others, depending upon the particular type of flower structure and behavior.

Previous studies indicated that plants of *Melilotus alba* will set seed freely without artificial manipulation of the blossoms and when the plants are screened to prevent pollination by insects, whereas *Melilotus officinalis* sets almost no seed when the blossoms are undisturbed by artificial means. We now believe that these findings require considerable modification, since our studies have shown that both the white and yellow blossom species of *Melilotus* contain strains which are naturally self-fertilized. We have also learned something of the mechanism which is responsible for differences in seed setting under controlled conditions of pollination. Results of these studies are given in the following pages, together with a discussion of their significance in relation to methods of breeding better varieties of sweet clover.

Literature Review

Kirchner (4) enclosed flowers of *M. alba* in gauze bags and concluded from a limited study that spontaneous self-fertilization was of general occurrence. Coe and Martin (1) conducted an extensive series of pollination studies with *M. alba*. In their experiments the flowers were enclosed in tarlatan, a material which has a weave twice as close as ordinary gauze. These investigators

[1] *Manuscript received June 12, 1931.*
Contribution from the Field Husbandry Department, University of Saskatchewan, Saskatoon, Sask.

[2] *Professor of Field Husbandry, University of Saskatchewan.*
[3] *Research Assistant, University of Saskatchewan.*

obtained less than one pod per raceme from protected plants, and concluded that "spontaneous self-pollination does not occur regularly as stated by Kirchner".

Kirk (5) studied the question of spontaneous self-fertilization in both *M. alba* and *M. officinalis*. The plants were completely enclosed in cages three feet square and five feet high, made of medium weight cotton with a weave of 52 strands to the inch. *M. alba* produced an average of 34.9 pods per raceme when enclosed in cotton cages as compared with 66.4 pods per raceme for open-pollinated plants. *M. officinalis*, on the other hand, gave only 2.6 pods per raceme as compared with 63.9 pods for open-pollinated plants. The difference between the two species was very pronounced, and the seed setting of protected plants was consistently in favor of *M. alba*. It was concluded from this study that *M. alba* will set seed freely without artificial manipulation or the visitation of insects, but that *M. officinalis* will produce very few pods when insects are excluded.

More recently the results of pollination studies with sweet clover have been published by Dann (2) and Ufer (8). Dann used one paper bag to enclose several racemes on each of 10 plants of *M. alba*. In two bags no pods were obtained, the other plants giving 4.0, 8.5, 9.0, 9.2, 18.2, 21.1, 59.0 and 60.0%. He assumes in the last two cases that small insects may have gained access to the flowers, and concludes that *M. alba* is capable of natural self-fertilization to a slight degree only, but entirely self-fertile on artificial selfing. *M. officinalis*, on the other hand, produced no pods in any of the bags and was therefore judged to be incapable of seed setting without artificial manipulation.

Ufer studied the question of seed setting in *Melilotus* by protecting the plants from insect visitation with pergamin bags of two sizes, both with and without ventilation. Seven biennial and five annual species and varieties were included in his experiments. Owing to unfavorable weather conditions and the possible effects of unnatural conditions within the bags, the author did not consider that a true picture of the actual facts had been obtained. At the same time he was reasonably sure of certain general conclusions which we have taken the liberty of summarizing as follows: There is a tendency for spontaneous self-fertilization in the genus *Melilotus* to vary with the individual plant. Some species favor autogamy more than others. In general, *M. alba* and *M. coerulea* are more pronounced self-fertilizers than *M. officinalis*. No seed was obtained by selfing *M. dentatus*, *M. suaveolens*, and *M. infestus*. Good seed setting was always obtained by selfing *M. wolgicus*, *M. polonicus* and *M. indicus*. With *M. altissimus*, *M. italicus* and *M. sulcatus* the results varied between plants within each species. Within single plant progenies the results are much more uniform than between unrelated plants.

Self-fertilization in M. alba

Observations over a period of years have given the writers considerable evidence that at least some varieties and strains of *M. alba* consist of a mixture of naturally self-fertilizing and naturally cross-fertilizing plants. These observations may be stated as follows:

(1) When single plant progenies from open-fertilized seed are grown and the plants compared, some of the families show very little variability.

(2) When single plant progenies from first generation self-fertilized seed are grown, a certain percentage of the lines possess a high degree of uniformity in plant type, while others are extremely variable.

(3) When single plants are caged to exclude insects, some plants produce much more seed than others. This fact may be seen in the data presented by Kirk (5), Dann (2), and Ufer (8).

(4) Plants grown in a greenhouse which has been thoroughly screened to exclude insects are so variable in seed setting that some of them produce no seed at all, while others produce a pod for practically every flower. We have observed this fact for three seasons.

During the past two years selfed seed has been taken from plants which are able to produce self-fertilized seed spontaneously in different degrees, and progenies from these individuals were grown in order to study the genetics of this character. At this time it is necessary to submit only one piece of evidence to show that clear cut segregation takes place.

In the fall of 1929 sweet clover plants were taken into the greenhouse for various purposes. Among these were two plants from a first generation selfed line. When these blossomed during the winter, one plant produced a large amount of seed by spontaneous self-fertilization, while the other produced no seed except by manipulation of the flowers. Subsequent observation with the other 32 plants of this line showed that these had segregated for this character.

TABLE I

PODS PRODUCED BY HIGH AND LOW SEED SETTING PARENT PLANTS FROM A SINGLE SELFED LINE OF *M. alba*

Plant No.	No. of racemes	Total pods produced	Pods per raceme	Seed setting, %
High seed setting parent plant				
1	18	510	28.34	41
2	17	626	36.82	54
3	15	505	33.67	50
4	16	532	33.25	49
5	21	750	35.71	52
6	15	504	33.60	49
7	13	545	34.23	50
8	21	1395	66.43	98
Low seed setting parent plant				
9	21	3	0.14	0.2
10	21	0	0.00	0.0
11	10	0	0.00	0.0
12	19	6	0.32	0.5
13	19	1	0.05	0.1
14	21	6	0.29	0.4

Selfed seed was secured from the two plants mentioned above, and progenies from each were grown in the season of 1930. Because of limited greenhouse space only 14 plants could be studied in the greenhouse. Six of these came from the non-seeder and eight from the heavy seeder. The amount of seed setting by each plant is given in Table I.

It will be observed from Table I that the low seed setting parent produced only plants which were incapable of natural self-fertilization. On the other hand, the high seed setting parent produced one plant (No. 8) which was completely self-pollinated and naturally self-fertilized. Almost all of the flowers on this plant produced mature pods. The other seven plants of the same family were intermediate with respect to the number of seeds produced, the average being about 49%. From these observations and other data which are still incomplete, we concluded that the character of spontaneous self-fertilization in *M. alba* is inherited and that those plants were heterozygous which were intermediate in seed production.

The data presented in Table I have been selected from inheritance studies (which will be published later), in order to show that the character of natural self-fertilization in white sweet clover exhibits clear-cut segregation, and also because these plants were part of the material which was used in studying the mechanism by which self-fertilization is facilitated or prevented. The flower structure and behavior in relation to seed setting constitutes the chief topic of discussion in this paper, but before proceeding with the subject, it will be necessary to describe the remainder of the material which was studied. This includes several varieties of yellow blossom sweet clover, to which we shall refer briefly in order especially to record a particular case of natural self-fertilization in this species.

Self-fertilization in M. officinalis

While opinions have differed widely as to whether *M. alba* will or will not set seed without artificial treatment or the visitation of insects, all investigators, including the writers, have been agreed that M. *officinalis* is not spontaneously self-fertilized. It is of special interest, therefore, to report an exception to this rule. The variety in question is called "Redfield Yellow", because it was developed at the U.S.D.A. plant breeding station at Redfield, South Dakota. That the plants produce seed by being naturally self-fertilized does not appear to have been reported by those who have given it prominence. This variety is being grown quite extensively at the present time in western South Dakota and adjacent states.

When Redfield Yellow sweet clover was first grown by us about four years ago, we were surprised to see the distinctive appearance and uniformity of plant type. This was observable in the general appearance of stems and foliage, its very late maturity, and characteristic appearance of the inflorescence. The absence of variability in these characters was in sharp contrast to that of other varieties of the yellow flowered species. Not until plants were grown in the greenhouse and brought to maturity in the winter by the aid of electric lights did we observe that approximately 70% of the flowers set seed without artificial manipulation.

The other types of yellow blossom sweet clover which were used in this study included (1) Albotrea, a variety of the common American type, and (2) Zouave, which differs from common yellow sweet clover in producing an upright growth and retaining its foliage late into the fall of the first season. These two types will not produce seed unless the flowers are manipulated artificially.

That the different types of yellow sweet clover mentioned above should be referred to *M. officinalis* is not at all certain, but until more is known with respect to the taxonomy of the genus there seems no good reason for recognizing more than one species of biennial sweet clover with yellow blossoms.

Flower Structure and Pollination in Relation to Seed Setting

Having discovered self-fertilizing white-blossom sweet clover and strains which will not set seed by natural self-fertilization, as well as plants which are intermediate between these extremes, we were much interested in investigating the flower structure and mechanism which might be responsible for the differences in behavior. Having discovered also that Redfield Yellow sweet clover was naturally self-fertilized we were anxious to include in the study several yellow flowered varieties.

In order to provide a ready means of reference to the material which was studied, the different types and varieties have been assigned the following letters:

*A.—*M. alba.* Plants spontaneously self-fertilized, the flowers of which nearly all produce pods (Table I, plant 8).

**B.—*M. alba.* Plants do not produce seeds normally by self-fertilization (Table I, plants 9 to 14).

**C.—*M. alba.* About 50% of the flowers produce pods by spontaneous self-fertilization (Intermediate types, Table I, plants 1 to 7).

D.—*M. officinalis* (Variety, "Redfield Yellow"). Plants spontaneously self-fertilized.

E.—*M. officinalis* (Variety, "Albotrea"). Plants do not produce seeds normally by spontaneous self-fertilization.

F.—*M. officinalis* (Variety, "Zouave"). Plants do not produce seeds normally by spontaneous self-fertilization.

In each of the types and varieties (A to F) large numbers were carefully examined in order to determine, if possible, the factor or factors which influence seed setting. We hoped, then, to be able to relate this information to what was known about the actual percentage of seed production under controlled conditions of pollination. Having removed the petals, flowers were observed singly under the microscope.

The results of this study, in which several plants of each type and thousands of flowers were examined, proved very instructive. Several details of flower structure and behavior were noted which appeared to be significant as affecting the tendency of certain types to produce seed by spontaneous self-fertilization

**A, B and C plants belonged to a single first generation selfed line.*

and the actual percentage of pods produced. These observations had to do chiefly with the relative position of anthers and stigmas and the quantity and condition of the pollen. For a long time it seemed impossible to construct any kind of picture which could be related consistently to seed setting percentages of the different types of sweet clover. Besides, information was lacking as to the exact stage of flower development at which the anthers dehisce, because it was not certain whether the anthers had been disturbed when the petals were being removed, resulting in premature liberation of the pollen.

At this stage the idea occurred to us of attempting to render the petals transparent by treating the flowers with some substance that would act as a clearing agent. After many trials with different preparations, a method was devised which was found to be perfectly satisfactory. It was successful not only with white flowers but also with yellow flowers, making the position of stamens, style and pollen grains clearly visible. In the case of yellow petals, most of the coloring disappeared, while the natural color of anthers and pollen was not appreciably affected.

When it is desired to render the flowers transparent in order to observe the normal internal condition at any stage of development, the standard is first removed with a pair of tweezers and the two wing petals clipped off about halfway down. Each flower is then placed on its side on a glass slide. A large number of flowers may be arranged in series and treated at one time. The flowers are then saturated with absolute alcohol for 15 min. by applying one drop at a time to each as often as is necessary. They are then treated for 30 min. with sufficient cedar clearing oil to cover them, and observations are made through the oil. A magnification of 60 was used in these experiments.

With this improvement in technique it was now possible to extend our information to include the exact stage of flower development at which the anthers normally discharge their pollen. The natural distribution of pollen also, and its position in relation to the stigma, was clearly evident. These facts proved to be of first importance. So successful indeed was this method of examination that we decided to duplicate all previous observations and measurements.

Several factors have been referred to already which may conceivably affect seed setting in sweet clover, and there are still others, not yet described, which may be added to the list. These will now be considered separately, and as far as possible an attempt will be made to interpret the behavior of each variety with respect to seed production in terms of the special combination of factors which it exhibited.

Position of stigma in relation to the anthers. The stamens in different plants of *M. alba* and in plants representing different varieties of *M. officinalis* were found to vary greatly in length. In flowers with short stamens the style protruded a considerable distance beyond the highest anther: This condition was typical in the case of all plants designated B. These produced practically no pods unless the flowers were manipulated artificially. "A" plants of the same selfed line had long stamens, the anthers were as high as the stigma,

and practically all flowers produced pods. "C" plants of the same selfed line had stamens of intermediate length, and all of these plants produced about 50% of the possible number of pods (see Fig. 2). So far as *M. alba* is concerned, therefore, this character appeared to have some significance in the material which was studied.

In order to show the degree of variability within each of the types A, B and C in *M. alba*, measurements were made on 100 flowers of each plant. Instead of measuring the length of the filaments, which presented certain difficulties, the more significant determination was made of the distance from tip of style to highest anther. This information is presented graphically in Fig. 1. The curve (A) for flowers with long stamens would have been as symmetrical as the others had not the anthers in many of the flowers protruded beyond the style.

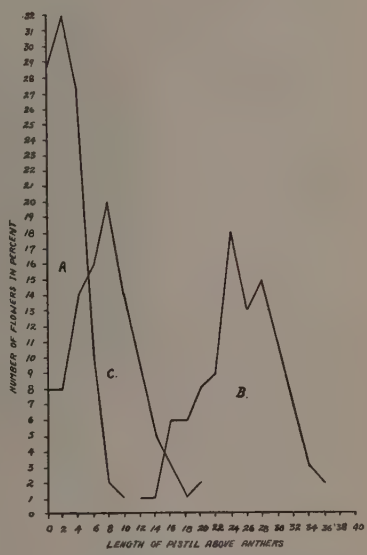


FIG. 1. *Class frequencies (based upon length of styles projecting beyond anthers) in flowers of plant segregates in a selfed line of white sweet clover (Melilotus alba). (A) Naturally self-fertilized flowers with long stamens. (B) Flowers with short stamens and not naturally self-fertilized. (C) Flowers with stamens of intermediate length. About 50% were naturally self-fertilized.*

With respect to varieties of *M. officinalis* which were studied for length of stamens, the condition was very interesting, since there was no such consistent relation to seed production as was found in *M. alba*. Redfield Yellow, which produces a high percentage of pods by natural self-fertilization, had very short filaments similar to the "B" type plants of *M. alba*. On the other hand, plants of the variety Albotrea had very long filaments so that the anthers were adjacent to the stigma and frequently they were above it. In this respect these corresponded to the "A" type plants of *M. alba*, but unlike the latter they failed to produce pods when the flowers were undisturbed. The third variety, Zouave, had very short stamens and did not produce pods by natural self-fertilization. This variety, therefore, did not violate the rule which seemed to apply for the plants of *M. alba* which were studied.

With respect to the relative position of anthers and stigma, we can say only that in the plants of *M. alba* which were studied the close proximity of anthers and stigma seemed to facilitate self-fertilization, but that this assumption did not hold as far as *M. officinalis* was concerned. Obviously, there must be other factors which play a part.

Stage of flower development when the pollen is liberated from the pollen sacs. In all of the material which was studied it was found that at least some of the pollen sacs had discharged their pollen within the flower just preceding the

unfolding of the bud. In other words, one or two and sometimes three or four unopened flowers, immediately above the last blossom to expand, were found almost invariably to contain some free pollen. Obviously this fact only served to complicate the problem. It is interesting to note that its discovery could not have been arrived at except by the method of making the petals transparent. For reasons which need not be stated it is certain that the treatment did not contribute to the observed result.

Distribution of pollen at the time when the flowers expand. Careful observations were made on all of the material in order to determine the exact distribution of pollen within the flower. For this purpose the last blossom to open on the raceme was chosen. In other words, the flowers selected were those only which are the last to open and immediately adjacent to the unexpanded buds.

The observed distribution of pollen within the flowers, taken in conjunction with determinations on the relative position of anthers and stigmas, appears to be very significant, and throws a good deal of light on the problem of spontaneous self-fertilization in sweet clover. Considering first the A, B and C type plants of *M. alba*, it was found that the percentage of flowers having pollen on the stigma was in exact agreement with the percentage of pods formed, the latter being based on total number of flowers. Observations were made on 100 flowers per plant. The stigmas of "A" type plants invariably were covered with pollen. In about 50% of the cases, flowers of "C" type plants had pollen on the stigmas. Very rarely, however, was it possible to find flowers on "B" type plants in which a single pollen grain was found on the stigma. In this connection it should be stated that all three types would set seed freely when the flowers were artificially manipulated.

In the yellow flowered species only two of the three varieties, namely Redfield Yellow and Zouave, showed the same exact agreement between seed setting and distribution of pollen which was observed in *M. alba*. In Redfield Yellow, approximately 70% of the flowers had pollen grains on the stigma and the percentage of pods was about the same. In no case was pollen found on the stigmas in flowers of the variety Zouave, and no seed was produced by natural self-fertilization. But Albotrea, in this case, proved to be the exception to the rule, since this variety produced no pods, although pollen had been deposited on the stigma in 95% of the cases. With respect to distribution of pollen, therefore, it may be stated that a perfect correlation exists in five of the types and varieties which were studied between percentage of seed setting and percentage of flowers which had pollen grains in contact with the stigmas. The Albotrea variety proved to be a notable exception in this respect.

Fig. 2 shows the average condition which was found in flowers of the different plant types which were studied with respect to distribution of pollen as well as the relative position of stamens and styles. The drawings were made with a camera lucida attachment and with a magnification of about 120 diameters.

Size of keel. Differences were observed with respect to flower size in different varieties. Size of flower determines the amount of space within the upper portion of the keel above the anthers. The relative sizes are indicated in Table III. This factor appeared to have significance in the case of Redfield

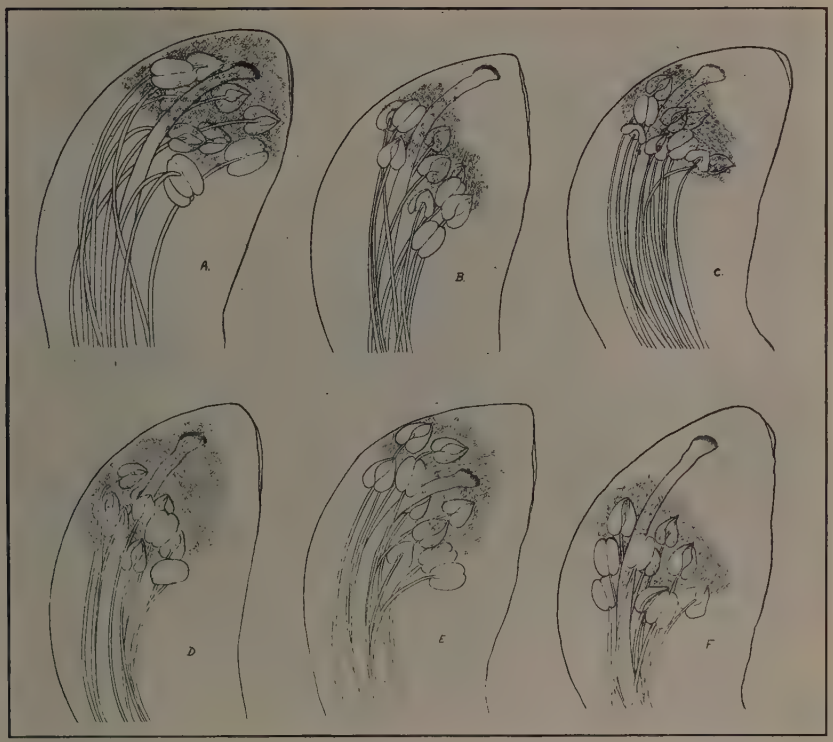


FIG. 2. *Camera lucida drawings showing the average condition within the keel when sweet clover flowers first open with respect to length of stamens and distribution of pollen. A, B, C—Plant segregates in a selfed line of M. alba. D, E, F—Varieties of M. officinalis; Redfield Yellow, Albotrea, and Zouave, respectively. A and D are naturally self-fertilized. B, E and F will not set seed unless the flowers are manipulated. C is naturally self-fertilized in about 50% of the cases.*

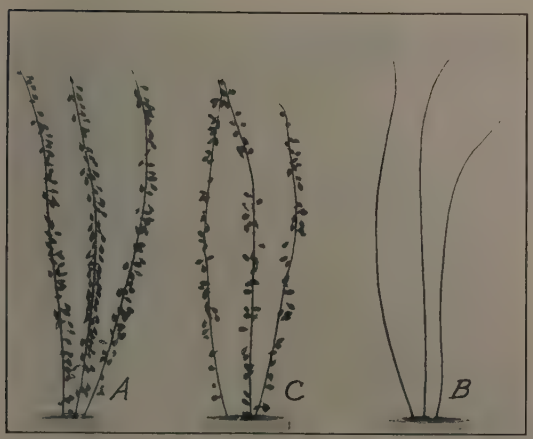


FIG. 3. *Racemes from plant segregates in a selfed line of white sweet clover, M. alba, showing the typical numbers and distribution of pods at maturity. (A) Naturally self-fertilized. (B) Not naturally self-fertilized. (C) Fifty per cent of the flowers naturally self-fertilized.*

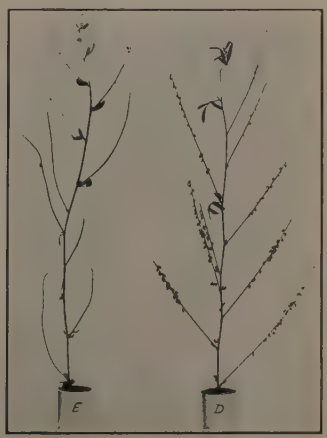


FIG. 4. *Yellow blossom sweet clover, M. officinalis, showing seed setting in two varieties. (D) Redfield Yellow; (E) Albotrea.*

Yellow sweet clover, since it appeared to be the only explanation why the pollen mass, in flowers of this variety, was nearly always heaped up beyond the anthers until the highest pollen grains came in contact with the stigmas. The mass of pollen grains always gave the appearance of having been pushed upwards in close quarters. Whether this is the explanation of what actually takes place, it is impossible to say, but the peculiar distribution of pollen in this variety was very characteristic (see Fig. 2).

Amount of pollen. This is a factor which appears to be of considerable importance if we may judge by the striking differences which were found between the different types and varieties which were studied. As between flowers and plants within each type, there was very little variation.

Plants of *M. alba* which produced seed by natural self-fertilization ("A" type, with long stamens) invariably had flowers with large amounts of pollen. "C" type plants of *M. alba*, which were intermediate with respect to length of stamens and seed setting, in general produced an intermediate quantity of pollen. "B" type plants of *M. alba*, which had short stamens and did not produce seed by natural self-fertilization, invariably produced a very limited supply of pollen.

In the yellow flowered species, Redfield Yellow, with short stamens and high seed setting, had a large quantity of pollen. Zouave and Albotrea varieties, on the other hand, with very short and very long stamens respectively and neither of which produced seed by self-fertilization, were intermediate with respect to quantity of pollen.

With reference to quantity of pollen, therefore, it may be said that large quantities were found to be closely associated with high seed setting, and small amounts with low seed setting, under the conditions of the experiment. Quantity of pollen was not associated with length of stamens. It was not possible in these studies to attach any special significance to this factor, but the facts were so striking as to be of special interest, and call for further study.

Condition of pollen. Perhaps the most interesting observation which was made in connection with spontaneous self-fertilization in sweet clover had to do with germination of the pollen grains in the flower. During the investigation it was early observed that when flowers were tripped with the flat end of a wooden toothpick certain plants discharged a large quantity of pollen, the grains of which tended to cling together in a mass. This was characteristic of "A" type plants of *M. alba* and of the variety Redfield Yellow, both of which produced an exceptionally high percentage of pods. The other four types studied produced either a medium or very small amount of pollen, and this was always dry and powdery. These two conditions of pollen gave the impression that some plants produced sticky pollen and others, pollen which was just the reverse. Examination under the microscope, however, disclosed the fact that this tendency for the pollen grains to cling together in a mass was due to their having germinated. The mass consisted not only of pollen grains but of pollen tubes which served to hold them together.

It was discovered further (by observing the flowers after they had been rendered transparent) that in the two cases referred to above, the pollen grains

invariably had germinated *en masse* before the flowers had opened. This would seem to be a very remarkable adaptation to insure self-fertilization. We arrive at the conclusion, therefore, that plants of the "A" type in *M. alba* and plants of the Redfield Yellow variety in *M. officinalis* are completely and spontaneously self-fertilized. Cross pollination by means of insects would seem to be a practical impossibility.

In the flowers of the other types of sweet clover which were studied, those having so-called "dusty" pollen, germinated pollen grains were never found. It should be stated that attempts to germinate such pollen grains in water were always successful, but only with a small proportion of them. This proved that they did not altogether lack viability. Presumably the percentage germination would have been quite satisfactory in a more favorable medium. This is being investigated.

Another point in this connection should be recorded because it strengthens the assumption that certain types of plants are normally self-fertilized, and because it is useful information in connection with artificial crossing in sweet clover. In the case of "A" type plants in *M. alba* and plants of the Redfield Yellow variety, both of which produced germinating pollen in the unopened flowers, it was practically impossible except by drastic methods to remove all of the pollen from the surface of the stigmas. On the other hand, flowers of plants which had "dusty" pollen could be taken after they had opened, the petals removed, and the stigmas completely freed from pollen by simply blowing on them. This may be done with the breath or by means of a dental syringe. What has been stated is especially true of the "B" type plants in *M. alba* which did not produce pods by natural self-fertilization. Pollen was rarely found in contact with the stigmas, even after the petals were removed from fully opened flowers. With these plants emasculation is a simple procedure which is likely to be highly efficient by any method. On the other hand, emasculation is very difficult and highly unreliable with any method in the case of those types where the pollen is germinated before the flowers open, unless the latter are taken at a very early stage of development.

Receptivity of the stigma. The problem still remained to be explained why the variety Albotrea of the yellow flowered species would produce practically no seed, although pollen grains were found in contact with the stigmas in 95% of the flowers, and this at an early stage of development before the petals had expanded. The same problem existed to a lesser degree with respect to "B" type plants of *M. alba* and plants of the variety Zouave, either of which would produce only a comparatively small amount of seed when the petals were removed and the stigmas dusted with pollen from the same plant. Since some of the pollen at least was known to be viable, it was natural to suspect that the stigmas in some manner were not receptive.

Plants which are not capable of spontaneous self-fertilization must depend for their fruitfulness on the visitation of insects. It is possible that when honey bees, which are the most important pollinators of sweet clover, visit the flowers, the stigmas are more or less scarified by coming in contact with the body of these insects. Since no other circumstance suggested itself which was

likely to affect the stigmas of sweet clover flowers, an experiment was planned to test the validitiy of this hypothesis.

Several racemes were selected on each of two plants of the three varieties in question, and one other plant of *M. officinalis* designated "X". The petals were carefully removed from a number of freshly opened flowers. On ten racemes of each type the flowers were dusted with pollen from the same plants, care being taken to make sure by examination with a lens that an abundant supply of pollen grains were adhering to the stigmas. Another group of ten racemes had the stigmas of each flower crossed back and forth with a small camel's-hair brush. The third group of ten racemes was treated by rubbing the stigmas with the flat end of a toothpick. Pollen was applied after each operation. The result of this experiment is presented in Table II, which gives the number of flowers treated and the number of pods produced in per cent for each treatment and each variety.

TABLE II

EFFECT OF SCARIFICATION ON RECEPTIVITY OF THE STIGMAS IN TERMS OF SEED SETTING IN VARIETIES OF SWEET CLOVER WHICH DO NOT PRODUCE SEED NORMALLY BY SPONTANEOUS SELF-FERTILIZATION

Variety	No. of racemes	No. of flowers	Method of scarifying the stigma	No. of pods produced	Per cent of flowers which produced pods
B type plants					
M. alba	10	125	None	19	15.2
M. alba	10	110	Soft brush	20	18.2
M. alba	10	90	Toothpick	64	71.1
Zouave					
M. officinalis	10	142	None	9	6.3
M. officinalis	10	141	Soft brush	15	10.6
M. officinalis	10	152	Toothpick	100	65.8
Albotrea					
M. officinalis	10	162	None	6	3.7
M. officinalis	10	158	Soft brush	7	4.4
M. officinalis	5	74	Toothpick	32	43.2
"X"					
M. officinalis	10	125	None	22	17.6
M. officinalis	10	115	Soft brush	37	32.2
M. officinalis	10	110	Toothpick	97	88.2

It is evident from the data presented in Table II that scarification of the stigmas with a toothpick was effective in making the stigmas receptive. The results were not very different for each of the three varieties. Apparently the treatment with a camel's-hair brush was not sufficiently severe to make a significant difference in the number of pods produced.

Seven factors have been described which appear to be more or less associated with the degree of spontaneous self-fertilization in sweet clover. These are tabulated in Table III in order to show the precise combination of characters which were characteristic of the plants in each of the types and varieties which

were studied. A large number of flowers on each plant were examined, but the number of plants in each variety was necessarily very limited. Those which were studied happened to possess the same characteristics, but it is very improbable that these are the only combinations of factors which would have been found if a larger number of individual plants had been examined.

TABLE III

FACTORS WHICH APPEAR TO INFLUENCE THE DEGREE OF SPONTANEOUS SELF-FERTILIZATION IN TYPES AND VARIETIES OF SWEET CLOVER

Character	Types of *M. alba*			Varieties of *M. officinalis*		
	A Self-fertilized	B Not self-fertilized	C Intermediate	D Redfield Yellow. Self-fertilized	E Albotrea. Not self-fertilized	F Zouave. Not self-fertilized
1. Percent seed setting	98	0.05	49	70	None	None
2. Length of stamens	Long	Short	Intermediate	Short	Long	Short
3. Liberation of pollen	Unopened flower	Unopened flower	Unopened flower	Unopened flower	Unopened flower	Unopened flower
4. Distribution of pollen	On stigma 100%	On stigma 0.25%	On stigma 50%	On stigma 70%	On stigma 95%	On stigma 0.0%
5. Size of keel cavity	Small	Small	Small	Very small	Large	Very large
6. Amount of pollen	Abundant	Scarce	Medium	Abundant	Medium	Medium
7. Condition of pollen*	Germinated	Not germinated	Not germinated	Germinated	Not germinated	Not germinated
8. Receptivity of stigma*	Receptive	Not receptive	Receptive	Receptive	Not receptive	Not receptive

**Data in these cases were obtained at an early stage of development just at the time when the flowers first open. In types A and D the pollen grains were germinated before the flowers opened.*

Discussion

The most significant fact brought out by this investigation is that certain plants of sweet clover are normally self-pollinated and spontaneously self-fertilized. In some cases cross-pollination is impossible, or at least negligible. It is fairly clear also that those peculiarities which characterize a plant of this type are inherited, and may therefore be utilized in breeding. The information which we have at the present time seems to indicate that the essential factors may be transmitted in a comparatively simple manner.

The significance of complete spontaneous self-fertilization as an inherited character in sweet clover is very important and quite obvious. Improved new varieties which are homozygous for this character may be grown without

danger of contamination with foreign pollen, and the superior qualities which they possess can be reproduced indefinitely, as is the case with most cereals. Indeed, the percentage of natural crossing may be less than in some cereals.

Natural self-fertilization in sweet clover will be a great asset in breeding for disease resistance. This crop is vulnerable to a fairly large number of destructive pathogenes, and it is becoming more and more evident that breeding for disease resistance will be one of the major problems with this crop. If selfed lines which are completely self-fertilized can be secured without too much effort, the task becomes very much less difficult. It is one thing to produce disease resistance, but quite another to retain the advantage of such a quality after it has been secured.

We have believed for a long time that plants which are naturally self-fertilized are of fairly frequent occurrence in *M. alba*, for the reasons stated earlier in this paper. Definite information has now been submitted to show that such plants do occur. The proportion of such plants will doubtless vary with different varieties of the white flowered species, but our observations suggest that spontaneous self-fertilization is associated more especially with the mid-tall, early maturing sorts such as are represented by the varieties "Arctic" and "Grundy County". There is reason to believe that the "Arctic" variety contains a considerable proportion of naturally self-fertilizing plants. Several of these have been isolated.

In view of the enormous advantage which a self-fertilizing variety would have from the standpoint of pure seed production, it would seem that plant breeders who are working with this crop should fully canvass the possibilities of spontaneous self-fertilization with the ultimate ambition to make all new productions homozygous for this character.

So far as *M. officinalis* is concerned, spontaneous self-fertilization seems to be of rare occurrence. During many years' work with several distinctive sorts of yellow flowered sweet clover, we found no indication that plants with this character exist until it was observed in the variety "Redfield Yellow".

Preliminary observations on the factors which appear to influence self-fertilization in sweet clover were made in connection with crossing work when the flowers were being emasculated by the "suction" method. This method was described by Kirk (6) and consists of removing the petals of fully opened flowers, after which the pollen is removed by suction through a small glass nozzle, the latter being attached by a rubber hose to an aspirator pump. The "suction" method has been criticized recently by Hackbarth (3) as being less effective than the water-jet method for emasculating alfalfa described by Oliver (7). It would appear that the facts reported herein offer a satisfactory explanation for the divergent results obtained by different investigators and show why the suction method can be very successful with certain types of plants but quite unsuccessful with others.

With respect to ease of emasculation, at least several different plant types may be distinguished as follows: (1) Plants with short, medium or long stamens, "dusty" pollen and unreceptive stigmas. These can be emasculated with ease and a high degree of certainty by almost any method. Very satisfactory

results were obtained by simply removing the petals and blowing off the pollen with a forced breath. (2) Plants with short stamens, "dusty" pollen, and receptive stigmas. With these the "suction" method is quite successful because the pollen grains do not come in contact with the stigmas unless the flowers are manipulated. In these cases also, a forced breath will remove the pollen grains quite effectively, and the "selfs" will be few in number. (3) Plants with long stamens, "dusty" pollen and unreceptive stigmas. These can be emasculated successfully with air or water pressure. If the "suction" method is used great care must be taken not to injure the stigmas, because if this takes place pollen grains may become strongly attached and self-fertilization is likely to occur. (4) Plants with long stamens and receptive stigmas. Whatever method is used, plants of this type must be emasculated at a very early stage of development before the flowers open. In these cases the "suction" method is of little value.

The difficulty of successful emasculation in the fourth type listed above is still greater if the pollen grains normally have germinated before the flowers open. Not only the "suction" method, but also the water-jet method and emasculation with a needle have proven very inefficient in our experiments with plants that possess these peculiarities. Germinated pollen adheres to the stigma much more tenaciously than "dusty" pollen, presumably because they are held fast by germ tubes which have penetrated the tissues.

In our original experiments with the suction method we were unaware of the differences in flower structure and behavior which exist between different plants of sweet clover. The data which were presented (6) seemed to be sufficiently conclusive to justify a favorable report. On the other hand, all of the experiments were made with a comparatively small number of plants and these may have included only types (1) and (2), and not types (3) and (4).

Acknowledgment

The authors which to express their thanks to Mr. Frank Rose, Field Assistant, who has contributed much to the success of this investigation both in the field and in the laboratory.

References

1. Coe, H. S. and Martin, J. N. Sweet clover seed. U.S. Dept. Agr. Bull. 844. 1920.
2. Dann, B. Uber die Befruchtungsverhaltnisse der Bastardluzerne (*Medicago media*), anderer *Medicago*-Arten und Steinklee (*Melilotus*). Z. Pflanzenzuchtg. 15: 366-418. 1930.
3. Hackbarth, J. Künstliche Kreuzungsmethoden bei Steinklee und Luzerne. Der Zuchter, 2: 354-358. 1930.
4. Kirchner, O. Uber die Wirkung der Selbstbestanbung bei den Papilonaceen. Naturwissenschaften, 3: 1-16. 49-64, u. 97 bis III. 1905.
5. Kirk, L. E. Self-pollination of sweet clover. Sci. Agr. 6: 109-112. 1925.
6. Kirk, L. E. Abnormal seed development in sweet clover species crosses. A new technique for emasculating sweet clover flowers. Sci. Agr. 10: 321-327. 1930.
7. Oliver, C. V. Alfalfa seed production: Pollination studies, U.S. Dept. Agr. B.P.I. Bull. 75. 1914.
8. Ufer, M. Untersuchungen über die Befruchtungsverhältnisse einiger Melilotusarten (Steinklee). Der Zuchter, 2: 341-354. 1930.

CHEMICAL CHANGES IN NITROGEN FRACTIONS OF PLANT JUICE ON EXPOSURE TO FROST[1]

By R. Newton[2], W. R. Brown[3] and J. A. Anderson[4]

Abstract

Exposure of the press-juice of unhardened winter wheat plants to frost caused a decrease in coagulable protein content, an increase in amino nitrogen content and an increased sensitivity to hydrolysis by dilute sodium hydroxide. Added sugar reduced the amount of change. No change resulting from freezing could be demonstrated in the inorganic nitrogen fractions. It is suggested that protein splitting as found in hardened plants may be the result of frost rather than an adaptation against it, and that the value of sugar as a protection to winter plants may lie partly in its ability to delay this action.

Introduction

The splitting of proteins during the hardening process in the autumn to simpler, less easily precipitated forms has been held by Schaffnit (4) and Harvey (1) to be an adaptation of winter plants against frost injury. Harvey found that the amino-acid content of cabbage leaves increased on hardening, and concluded that the protein cleavage thus indicated was the principal effect of the hardening process. Though not expressly stated, the inference from his paper is that the plants analyzed were never exposed to frost during hardening in a cold-frame. The increase of amino nitrogen we have fully verified in work with winter wheat plants (2), but have observed that the most pronounced increase occurs after the advent of sharp night frosts.

In the course of work on the frost precipitation of the proteins of plant juice (3), it was noticed that the nitrogen fractions seemed to be slightly altered during exposure to frost. The questions arose as to whether the frost had caused some chemical change in the proteins, and whether protein splitting as observed in winter plants was an effect of frost rather than an adaptation against it. A closely related question concerned the possibility that the protective value of sugar might lie in its power to delay frost cleavage.

Experiments looking to an answer to these questions were carried out in 1925, and repeated in 1927 for the purpose of checking and extending the results.

Materials and Methods

The winter wheat plants used were grown in the greenhouse, to obtain tissue in an unhardened condition in which the proteins would be sensitive to frost. The press-juice was extracted and handled as previously described (3).

[1] *Manuscript received June 10, 1931.*

Contribution from the Department of Field Crops, University of Alberta, with financial assistance from the National Research Council of Canada.

[2] *Professor of Field Crops and Plant Biochemistry, University of Alberta.*

[3] *Formerly postgraduate student, University of Alberta.*

[4] *Junior Research Biologist, National Research Laboratories of Canada.*

In 1925, the nitrogen of the fresh juice and of the juice, with and without added sugar, which had been stored in an ice-salt bath at −7° C., was classified into four fractions:

(1) heat-coagulable protein nitrogen,
(2) non-coagulable organic nitrogen,
(3) nitrate-nitrite nitrogen,
(4) ammonia nitrogen.

Coagulable nitrogen was determined by pipetting triplicate 10-cc. samples of press-juice into test tubes, adding to each 1 cc. *N*/1 sodium acetate and *N*/1 acetic acid, and immersing in a boiling water bath for 30 min., with frequent stirring. The juice was then filtered and the coagulum washed with 150 cc. of hot, distilled water. The filter paper and contents were then transferred to a Kjeldahl flask and the nitrogen determined by the Kjeldahl-Gunning-Arnold method.

The combined nitrate-nitrite and ammonia nitrogen was determined on the filtrate, made up to 250 cc. in a Kjeldahl flask, by Strowd's modification of the Devarda method (5), but substituting caprylic alcohol for paraffin as an anti-foam agent. Paraffin occluded carbon, and made complete digestion of the residue impracticable.

Non-coagulable organic nitrogen was determined by the Kjeldahl-Gunning-Arnold method, on the residue from the Devarda distillation, in the same flask.

Ammonia was determined on a fresh sample of juice, by distilling after diluting with water to 250 cc. and adding 2.5 gm. of sodium hydroxide, as in Strowd's modification of the Devarda distillation. The nitrate-nitrite fraction was obtained by subtracting the ammonia value from the combined value noted above.

In 1927, the following modifications of these methods were made. Determinations of coagulable nitrogen were carried out in quadruplicate (instead of triplicate). The determination of non-coagulable organic nitrogen was discarded, and a determination of amino-acid nitrogen by the Van Slyke method, on an aliquot of the undiluted filtrate from the coagulable nitrogen determination, substituted as a better index of changes in organic nitrogen fractions. The combined nitrate-nitrite and ammonia fractions were determined as before by the modified Devarda distillation of the filtrate from the coagulable nitrogen determination, but the ammonia was determined on aliquots of the same filtrate instead of on samples of fresh juice. Also, the ammonia distillation was carried out by the Van Slyke method, at 20 mm. pressure and 45° to 50° C. for 30 min., the filtrate being first saturated with calcium hydroxide. The amounts of ammonia found by this method were so small that to measure them satisfactorily it was necessary to use 100 cc. aliquots of undiluted filtrate. These were obtained by refluxing 250 cc. of juice in a boiling water bath for 30 min. and filtering.

Results of 1925 Experiments

The two series of results obtained in 1925 are included in the first half of Table I. They show that exposure to frost caused a decrease in the coagulable

nitrogen and apparently also in the nitrate-nitrite nitrogen, with a corresponding increase in non-coagulable and ammonia nitrogen. The changes were less in the sugar-protected juice.

TABLE I

CHANGES IN NITROGEN DISTRIBUTION OF PRESS-JUICE ON FREEZING

Wheat variety and nitrogen fractions	Mg. nitrogen in 10 cc. juice		
	Fresh juice	Frozen 20 hr. at −7° C.	
		Juice+8% sucrose	Juice only
Minhardi, 1925			
Coag. protein nitrogen	44.5	43.3	42.3
Non-coag. organic nitrogen	11.5	13.3	13.5
Nitrate-nitrite nitrogen	11.5	12.0	10.7
Ammonia by distn. with NaOH	3.7	3.5	4.6
Fulcaster, 1925			
Coag. protein nitrogen	32.5	30.8	30.7
Non-coag. organic nitrogen	9.8	11.0	11.1
Nitrate-nitrite nitrogen	13.3	12.3	11.7
Ammonia by distn. with NaOH	2.5	3.5	3.6
Minhardi, 1927			
Coag. protein nitrogen	22.9	22.6	22.3
Amino acid nitrogen	2.44	2.61	2.75
Nitrate-nitrite nitrogen	14.71	14.27	15.31
Ammonia by distn. with NaOH	0.90	1.21	1.30
Ammonia by Van Slyke distn.	0.15	0.14	0.15
Turkey, 1927			
Coag. protein nitrogen	34.2	33.3	33.2
Amino acid nitrogen	3.05	3.30	3.63
Nitrate-nitrite nitrogen	14.15	14.50	13.44
Ammonia by distn. with NaOH	1.17	1.10	1.17
Ammonia by Van Slyke distn.	0.32	0.24	0.21
Fulcaster, 1927		Frozen 6 hr. at −7° C.	
Coag. protein nitrogen	20.4	20.8	20.4
Amino acid nitrogen	3.30	3.04	3.32
Nitrate-nitrite nitrogen	14.43	14.64	14.71
Ammonia by distn. with NaOH	1.03	0.82	0.81
Ammonia by Van Slyke distn.	0.11	0.09	0.10

The changes in the organic fractions were interpreted as evidence that protein splitting is at least partly the result of exposure to frost, rather than an adaptation against frost injury. The smaller changes in the presence of sugar suggested that the protective activity of the latter may be in part chemical. The apparent changes in the inorganic fractions were not looked for, and the experiments of 1927 included a critical examination of the methods which led to the earlier result.

It will be seen in the first half of Table I that if the organic fractions are bulked together and the inorganic fractions treated likewise, that no appreciable

change has taken place in the totals. What is required therefore is a verification of the protein splitting which would account for the change of a small part of the coagulable to the non-coagulable form, and an explanation of the apparent reduction of nitrate-nitrite to ammonia.

Results of 1927 Experiments

The results of the 1927 experiments substantiated the change in the organic fractions, in the two cases where the juice was frozen 20 hr. at −7° C. The coagulable protein fraction was slightly decreased by the freezing, and an increase in amino-acid nitrogen afforded direct proof of protein splitting. As before, the changes were less in the portions of juice to which sugar had been added.

In the third case, in which the juice was exposed to frost only 6 hr., the changes if any were not measurable by the methods used.

Before discussing the inorganic fractions, the methods of determining them will be examined. In Table II are given the data for determinations of the combined nitrate-nitrite-ammonia nitrogen, the ammonia nitrogen, and the nitrate-nitrite nitrogen by difference. The methods which yielded the results shown in the first two columns of figures, differed only that in the case of the first column the solution of 250 cc. was heated with a 1 gm. of Devarda alloy for 1 hr. before distillation. The concentration of sodium hydroxide was the same in both cases. These were the methods of 1925, but they were now applied to the coagulable nitrogen precipitate as well as to the filtrate and the fresh juice.

TABLE II

EFFECT OF HYDROLYSIS IN DETERMINATIONS OF AMMONIA AND OF NITRATE-NITRITE BY THE DEVARDA METHOD

Juice from leaves of Khapli wheat	Mg. nitrogen in 5 cc. juice		
	Nitrate-nitrite-ammonia	Ammonia	Nitrate-nitrite by difference
Coag. nitrogen precipitate	1.55	1.75	—
Coag. nitrogen filtrate	3.92	0.80	3.12
Total	5.47	2.55	2.92
Fresh juice	4.72	2.32	2.40

If the method actually determined only nitrate-nitrite nitrogen and inorganic ammonia, it would not be expected to find any in the precipitate, since all the salts should be washed into the filtrate. The data show that in fact nearly 70% of the ammonia found in the fresh juice was due to the hydrolysis of amide and amine groups in the coagulable protein. The discrepancy between the sum of the amounts found in the precipitate and filtrate, and that found directly in the fresh juice, may be due to changes in the composition of

the proteins during the boiling necessary to coagulate them or to unequal hydrolysis during heating with the alloy. In either case it emphasized the empirical nature of the method.

Returning now to the lower half of Table I, it will be seen that in 1927 the ammonia distillations were carried out by two methods: with 1% sodium hydroxide as in 1925, and by the Van Slyke method. The latter method is commonly supposed to determine all free ammonium salts but no organic groups. It showed no increase in ammonia as a result of freezing. Indeed the amounts found are so small they may well be due to slight hydrolysis during heat coagulation. Attempts to determine ammonia on the fresh juice by this method failed owing to the large amount of juice required and the consequent impossibility of preventing frothing by any ordinary antifoam methods.

The sodium hydroxide distillation, like the foregoing, was carried out on the filtrate from the heat coagulable protein (rather than on the fresh juice as in 1925), and in the Minhardi juice showed an increase in the ammonia fraction on freezing. It was not expected to find much if any increase in this fraction determined on the filtrate, since the data in Table II indicate most of the ammonia to have come from hydrolysis of the coagulated protein. As a measure of true ammonia the figures must of course be rejected, but together with the similar results in 1925 they may be accepted as further proof of the breaking down of proteins during freezing. This process sets free amide and amine groups which are hydrolyzed by the sodium hydroxide and determined as ammonia.

Since the concentration of the inorganic fractions was very small and the methods available far from perfect, it cannot be said with certainty that freezing caused no changes in them. It is clear, however, that the apparent changes found in 1925 were mainly due to the error involved in determining ammonia by distilling fresh juice with 1% sodium hydroxide. There was, as already pointed out, no change in the sum of the nitrate-nitrite and ammonia fractions, and since the nitrate-nitrite was found by difference, the establishment of relative constancy in the ammonia necessarily eliminates the apparent variability in both. But the fact that the apparent changes in the inorganic fractions were due in reality to frost-induced changes in the organic fractions, whereby amide and amine groups were exposed to hydrolysis in the subsequent determination of ammonia, is of significance from the point of view of frost effects.

Conclusion

The results on the whole seem to justify the conclusion that frost causes some cleavage of the proteins of wheat leaf juice, as reflected in a decreased content of coagulable protein, an increased sensitivity to hydrolysis by dilute sodium hydroxide, and an increased content of amino nitrogen. The experiments do not afford proof that amino nitrogen may not increase at hardening temperatures without frost, but indicate that the protein splitting held by some investigators to be an adaptation against frost may be partly at least merely the result of frost. It is to be noted also that in a recent paper (3) it

has been shown that precipitation of the proteins of press-juice, comparable to frost precipitation, may occur at 0° C. without freezing. The precipitating action of cold, with or without ice formation, may be connected with protein splitting under the same conditions.

The action of sugar added to the juice, in reducing the amount of change by frost, seems highly significant in view of the widely recognized role of sugars in plants as a factor in frost resistance.

In this laboratory we have found that protein splitting, as indicated by amino nitrogen content, occurs in the late autumn in equal degree in all varieties, without regard to their respective winter hardiness. This lends color to the view that it is the result of frost, since all varieties were exposed to the same weather conditions. Sugar accumulation is also the result of low temperatures, but in this case varieties exhibit different levels corresponding in a general way with their relative winter hardiness, thus suggesting that it is a true adaptation against frost.

References

1. Harvey, R. B. Hardening process in plants and developments from frost injury. J. Agr. Research, 15: 83-112. 1918.
2. Newton, R. and Brown, W. R. Seasonal changes in the composition of winter wheat plants, in relation to frost resistance. J. Agr. Sci. 16: 522-538. 1926.
3. Newton, R. and Brown, W. R. Frost precipitation of proteins of plant juice. Can. J. Research, 5: 87-110. 1931.
4. Schaffnit, E. Studien über den Einfluss niederer Temperaturen auf die pflanzliche Zelle. Mitt. Kaiser-Wilhelm Inst. Landw. Bromberg, 3: 93-144. 1910.
5. Strowd, W. H. The determination of nitrites and nitrates in plant tissue. Soil Science, 10: 333-342. 1920.

CATALASE ACTIVITY OF WHEAT LEAF JUICE IN RELATION TO FROST RESISTANCE[1]

By R. Newton[2] and W. R. Brown[3]

Abstract

The catalase activity of press-juice of winter wheat leaves collected from the field at any time during the late summer and fall was directly related to the winter hardiness of varieties.

Introduction

Catalase activity has been quite widely used as a measure of the activity or vitality of tissues. Heinicke (4) states with regard to apple leaf tissue, "it is very probable that the ability to decompose hydrogen peroxide is a more sensitive measure of the metabolic status of the tissue than the usual chemical analysis." Appleman (1) reported catalase activity to be closely correlated with respiratory activity in potato tubers, while Burge (2) concluded that it was associated with oxidative processes in the animal body. Leggatt (5) has recently investigated its relation to seed viability, and has contributed a good review of the literature.

Investigations on winter-hardiness (6, 7) have shown that sugar disappears gradually from the leaves of winter wheat plants during the winter, the rate of loss being somewhat greater in non-hardy varieties. Consequently, the protective resource represented by the sugar is diminished at a corresponding rate. The presumption is that the rate of loss is connected with the rate of respiration. At the time the experiments reported in this paper were carried out, suitable facilities for low-temperature respiration measurements were not available. However, on the evidence of other workers such as Appleman and Burge, cited above, there seemed reason to expect that the respiratory activity of the tissue might be reflected to some degree in the catalase activity of the press-juice. Accordingly a series of determinations was made on the press-juice of plants collected from the field at progressive dates and handled as already described (7). Of the three wheat varieties used, Minhardi is classified as winter-hardy, Turkey as semi-hardi, and Fulcaster as non-hardy.

Method

The determination of catalase activity was carried out quite simply, using a Van Slyke amino nitrogen apparatus, as suggested by Harvey (3). The leaves were dusted with precipitated calcium carbonate before grinding, so that acids destructive to the catalase might be neutralized as soon as liberated. The press-juice was diluted at once with 10 parts of distilled water, as in this dilution its activity will remain unchanged for many hours, whereas the activity of

[1] *Manuscript received June 13, 1931.*
Contribution from the Department of Field Crops, University of Alberta, with financial assistance from the National Research Council of Canada.

[2] *Professor of Field Crops and Plant Biochemistry, University of Alberta.*
[3] *Formerly postgraduate student, University of Alberta.*

undiluted juice gradually diminishes. A convenient quantity of the diluted juice, with sufficient water to make to 20 cc., was introduced into the deaminizing bulb through the top funnel, and 10 cc. of Oakland Dioxygen, neutralized with calcium carbonate, added through the side burette at the moment of beginning the determination. A constant rate of shaking was maintained by the use of an electric motor. The time required to liberate constant increments of oxygen was measured with a stop watch. All solutions employed were kept in a water-bath at 20° C., and the laboratory was maintained approximately at this temperature. Without special appliances it was found possible to restrict fluctuations in room temperature within 1° C.

TABLE I

CATALASE ACTIVITY OF PRESS-JUICE OF WINTER WHEAT LEAVES

Variety	Date 1923	Juice equiv. of diluted aliquot		Time in seconds to split 20 cc. increments of O_2 from 10 cc. Dioxygen					Total time for 100 cc.	Av. rate per gm. solid cc./gm./sec.
		Vol. cc.	Solids mg.	1st 20 cc.	2nd 20 cc.	3rd 20 cc.	4th 20 cc.	5th 20 cc.		
Seeding of July 16										
Minhardi	Sept. 6	1	140	61	37	23	27	33	181	3.95
Turkey	Sept. 6	1	125	78	48	—	—	—	—	(2.54)
Fulcaster	Sept. 6	1	100	99	51	—	—	—	—	(2.67)
Minhardi	Sept. 14	1	119	101	69	83	72	89	414	2.03
Turkey	Sept. 14	1	115	81	61	57	57	54	310	2.80
Fulcaster	Sept. 14	1	110	117	89	93	87	97	483	1.88
Seeding of Aug. 15										
Minhardi	Oct. 4	1	160	23	30	33	32	41	159	3.93
Turkey	Oct. 4	1	165	124	179	214	265	316	1098	0.55
Fulcaster	Oct. 4	1	120	170	145	230	240	361	1146	0.27
Minhardi	Oct. 25	½	92	33	35	37	37	38	180	6.04
Turkey	Oct. 25	½	86	40	43	50	56	57	246	4.73
Fulcaster	Oct. 25	½	64	84	86	85	89	105	449	3.48
Minhardi	Nov. 16	½	128	18	27	34	39	43	161	4.85
Turkey	Nov. 16	½	89	26	33	37	40	46	182	6.17
Fulcaster	Nov. 16	½	84	65	61	61	67	71	325	3.66
Minhardi	Dec. 14	½	128	24	38	48	54	62	226	3.46
Turkey	Dec. 14	½	132	46	86	79	109	129	449	1.69
Fulcaster	Dec. 14	½	140	46	80	86	71	95	378	1.89

Results

In addition to reporting the time readings directly, in Table I, the average rate for the whole volume of 100 cc. (or 40 cc. in two cases) expressed as cc. oxygen per gm. solids in juice per sec., is also given. The time readings are of course inversely proportional to the activity of the sample of juice. The wheat varieties are arranged in the table in the order of their winter hardiness, so that the relation between activity and hardiness may be seen at a glance.

With two exceptions (Turkey, Sept. 14, and Fulcaster, Dec. 14) the results show in all collections a direct correlation between the catalase activity per cc. juice and winter hardiness. With two further exceptions (Fulcaster, Sept. 6, and Turkey, Nov. 16) this is also true on the basis of activity per gm. solids in juice. As the season progressed, the activity of the juice increased very markedly, so much so that it was necessary to reduce the aliquots used from the equivalent of 1. cc. juice to $\frac{1}{3}$ cc., to keep the evolution of oxygen at a manageable rate. This is largely due to the increasing concentration of the juice resulting from the reduction in moisture content which characterizes the hardening process. Since this moisture-reduction process is as a rule more marked in hardy varieties (6, 7), and their catalase activity per gram solids in juice is also more pronounced, it follows that the catalase activity of their tissues must be generally greater than that of non-hardy varieties.

The catalase activity fluctuates quite violently in different collections, possibly reflecting the influence of changes in weather conditions on the physiological activity of the plants. It is a point of considerable interest that the activity appeared to reach a peak in the collections of Oct. 25 and Nov. 16, just the period when sugar accumulation was approaching the maximum (7).

Assuming that catalase and respiratory activity are related, the results obtained would suggest a greater rate of respiration in hardy varieties. On the other hand, hardy varieties generally maintain their sugar reserves during the winter better than non-hardy varieties. These two statements can scarcely be reconciled without investigating the low-temperature respiration rates of the varieties concerned, a subject to be discussed in the next paper of this series. In the meantime it should be noted that, while some workers have found a relation between catalase and respiration, this is not universally admitted, some holding that catalase is rather a protective agent against excessive oxidation. It may easily be imagined that a heavy accumulation of such a readily oxidizable substance as sugar would be accompanied by a protective mechanism of the kind suggested. It should be noted, too, that while the plants were collected from the field where the temperatures were low, at least during the latter part of the work, the catalase measurements were made at 20° C. It was assumed that the catalase in all samples of juice would have the same temperature coefficient, and that the relation of the varieties would not be changed by working at ordinary room temperature. While there seems no reason to doubt this assumption so far as press-juice is concerned, in the actual cells of the living tissues other factors may be operative to change the relations at low temperatures. It should also be noted that catalase activity in certain cases, for example in resting seeds, is taken as an expression of vitality rather than activity, of potential rather than kinetic energy. This may perhaps be the case in leaves of winter wheat.

While too much stress should not be laid on the results of a single series of experiments, it is important to note that this very simple determination appears to provide a rather sharp index of the relative hardiness of varieties of winter wheat.

References

1. APPLEMAN, C. O. Relation of catalase and oxidases to respiration in plants. Maryland Agr. Exp. Sta. Bull. 191, 1915.
2. BURGE, W. E. Why chloroform is a more powerful and dangerous anesthetic than ether. Science, 46: 618-620. 1917.
3. HARVEY, R. B. Relation of catalase, oxidase, and H^+ concentration to the formation of overgrowths. Am. J. Botany, 7: 211-221. 1920.
4. HEINICKE, A. J. Factors influencing catalase activity in apple-leaf tissue. New York (Cornell) Agr. Exp. Sta. Mem. 62. 1923.
5. LEGGATT, C. W. Catalase activity as a measure of seed viability. Sci. Agr. 10: 73-110. 1929.
6. NEWTON, R. The nature and practical measurement of frost resistance in winter wheat. Univ. of Alta. Coll. of Agr., Res. Bull. 1, 1924.
7. NEWTON, R. and BROWN, W. R. Seasonal changes in the composition of winter wheat plants, in relation to frost resistance. J. Agr. Sci. 16: 522-538. 1926.

RESPIRATION OF WINTER WHEAT PLANTS AT LOW TEMPERATURES[1]

By R. Newton[2] and J. A. Anderson[3]

Abstract

Respiration measurements at −7°, 0° and 7°C. with four winter wheat varieties and two spring wheat varieties grown in the field, were carried on during the fall and early winter of four years. The rate of respiration in winter wheat decreased as the plants hardened. At the outset all varieties respired about alike. After hardening began, the rates at −7°C. were in the inverse order of hardiness, with the spring wheats faster than the least hardy winter wheat. As hardening progressed, the differentiation of the winter varieties at −7° C. became sharper, and could be observed also at 0° C., but at 7° C. very little if any relationship of this sort developed. The spring varieties, because of frost damage, could not be compared with winter varieties in the fully hardened condition, but their behavior early in the season confirms the general conclusion that at the freezing point or lower temperatures respiratory activity is inversely related to winter hardiness. This accounts for the observed fact that hardy varieties maintain their sugar reserves better than non-hardy varieties during the winter.

Introduction

The respiration of wheat plants at low temperatures was investigated in order to explain if possible the disappearance of sugar during the winter. The naturally occurring sugar concentrations in winter varieties had been studied for a number of seasons, and the results had amply confirmed the finding of other investigators that sugar content is directly related to frost resistance. This field of work has been reviewed quite recently by Åkerman (1). Experiments in this laboratory (4, 6) had also indicated that the value of sugar lay in its ability to retard or prevent the precipitation and denaturation of the cell proteins by frost; or by acid or salt concentration, the two factors commonly supposed to account for frost precipitation.

Maximum concentrations of sugar in winter wheat plants at Edmonton are usually found about the end of November, after which they fall off gradually. Hardy varieties have in general a higher concentration and a slower rate of loss. This suggested that they might have a more profound dormancy, characterized by a slower rate of respiration, and possibly also a more stable dormancy, and be less easily wakened into a more frost-susceptible condition of physiological activity by occasional warm periods during the winter.

Govorov (2) published in 1923 a study of the differences in respiration of winter and spring cereals. The carbon dioxide respired was determined by a Petenkoffer apparatus, the plants being placed in a large flask immersed in a darkened water bath. He found that at 21° C., a hundred plants of a spring variety of each of wheat and rye gave off slightly more carbon dioxide than a

[1] *Manuscript received June 16, 1931.*

Contribution from the Department of Field Crops, University of Alberta, with financial assistance from the National Research Council of Canada. The authors wish to acknowledge also the technical assistance of A. W. Saddington in carrying out the measurements of 1928.

[2] *Professor of Field Crops and Plant Biochemistry, University of Alberta.*

[3] *Junior Research Biologist, National Research Laboratories of Canada.*

corresponding lot of a winter variety. When the temperature of the bath was reduced to 0° C. for 12 hr. and then raised to 5° C., the differences between the spring and winter forms were increased. In a further experiment in which it was raised only to 3° C., the differences were still greater. No exact parallelism was found between the rate of respiration and winter hardiness. Presumably the differences he found between winter and spring varieties might be expected to exist in a lesser degree between hardy and non-hardy varieties of winter wheat.

In 1927, there appeared a report by Martin (3) on the respiratory rates of one spring and three winter wheats, and one winter rye, grown in pots in the greenhouse. The soil around the plants was sealed with a half-and-half mixture of vaseline and liquid paraffin, the plants covered with a vessel sealed at the edges with the same mixture, and the pots transferred successively to chambers at 5°, 0°, −5° and −10° C. At each stage, 8 to 12 hr. was allowed for the plants to come to equilibrium, after which air was passed through and the carbon dioxide evolution measured. The spring wheat did not survive the lowest temperature. Triplicate determinations and averages of these are given for each variety at each temperature, together with the van't Hoff coefficient for each variety between −10° and 0° C. He concludes that at −10° C. the rate per unit dry matter in the winter wheat varieties is in the inverse order of hardiness, and that the temperature coefficients of the winter wheats and rye are in the order of hardiness. A study of his table shows that the variations between replicates are so wide as to cast doubt on the exactness of the method, and his conclusions seem barely justified by the data.

The investigations of low temperature respiration reported in this paper were begun in the fall of 1925 and continued in the falls of the three succeeding years, being completed in December, 1928. The results reported here are confined to those obtained in the latter part of the third season and in the fourth season. Prior to this time, the apparatus and procedure were being continually modified and refined to meet technical difficulties which appeared one after another, as it became more and more apparent that the differences between varieties were very small and would require a precision of measurement which had not at first been anticipated. It is considered that the more accurate results obtained with the final apparatus are sufficient to establish the relationship under investigation.

The temperatures used were 7°, 0° and −7° C. These were selected partly because they made a substantial range and also fell conveniently within the capacity of our equipment, and partly because −7° C. appeared to be a point of special physiological significance. It is the temperature at which absolute killing of unhardened greenhouse tissue occurs and the point of maximum frost precipitation of the press-juice proteins (4). A series of varieties of graded resistance to frost were used, to ascertain the relation between respiratory activity and hardiness.

Materials

One of the greatest limitations to precision in biological work is the plastic, almost fluid, nature of the experimental material. It was considered essential

that in order to make the results comparable with the behavior of wheats under normal winter conditions in the field, the respiration measurements should be made upon plants grown in the field and hardened naturally by exposure to fall frosts. This greatly increased the difficulties. Slight differences in environment in different parts of a field plot, or changes in conditions from day to day, or even hour to hour, are reflected in the physiological activity of the plants. Varietal differences in low-temperature respiration are small, and the natural variation between two groups of plants of the same variety may be as great as the average difference between two varieties. It was sought to overcome these errors by collecting plants at the same hour every day, by running determinations on all varieties at the same time, and by repeating the experiments often enough to produce reliable averages.

Another important difficulty arising out of growing the plants in the field is that of obtaining uninjured tissues. It is well known that bruising and cutting increase the rate of respiration. In the Edmonton climate, the plants begin to harden with the first frosts about the middle of September. By the middle of October they are approaching their winter equilibrium, but when November comes, a cold snap, in which the temperature drops to 0° F. or below, is liable to occur. Such conditions, while not killing the plants entirely, seriously impair their usefulness for respiration measurements, since in all but the hardiest varieties the majority of leaves are killed, and even in the hardy varieties all but a few of the youngest leaves are frozen back for an inch or two from the tips. If snow comes early, the plants may be protected from both rapid changes in physiological condition and from freezing back at the tips of the leaves, but in brushing off the snow it is difficult to avoid some injury to the plants, especially in cold weather when the leaves are brittle with frost, or if an earlier thaw has caused the snow to freeze to the leaves. Without special measures, therefore, the period during which reliable results may be obtained is generally limited to a few weeks between the time when the plants become relatively hardened and the first severe frosts.

In 1928, the last season of this work, it was proposed to grow the plants in the field in boxes, these to be removed before danger of severe frost to a greenhouse kept at 0° C. When it became clear that the controlled greenhouse then in course of erection would not be ready in time, the varieties were seeded in the field in the usual way, in rows 1 ft. apart. Boards, 6 in. wide, were placed on edge between every fourth row, so that light burlap (Hessian cloth) could be stretched over the plants as a protection against snow. This was done every night after Sept. 28. Each morning the covering was rolled back so that the plants were exposed to normal light conditions. It so happened that there was no appreciable snowfall that season during the period of the experiments, but the covering doubtless retarded the freezing back of the tips of the leaves in the non-hardy varieties.

In all experiments reported in this paper, field-grown plants were used, from which the roots had been removed, all dead or wilted leaves stripped, and dead leaf-tips cut off slightly above the boundary of the living tissue to avoid injuring the latter.

The removal of the roots seems justifiable, since the leaves are the organs which determine winter survival in wheat. Killing begins at the tips and extends gradually downwards. If all leaves are killed to the base, the plant fails to survive. In this respect the cereals differ from legumes such as clover and alfalfa. In the latter, the roots and the crown buds are the critical points, the fall-grown leaves always dying off in the winter in this climate.

Four varieties of winter wheat and two varieties of spring wheat were used in these experiments. The winter varieties were Minhardi, Turkey, Fulcaster and Squarehead's Master, named in descending order of winter hardiness as found in our own experimental plots and elsewhere. For the purpose of the present experiments, the first two may be classified roughly as hardy and the other two as non-hardy. The data for the survival of these varieties at Edmonton during the five years 1924-29 are given in Table I. The respiration experiments extended over the last four years of this period.

TABLE I

PERCENTAGE SURVIVAL OF WINTER VARIETIES AT EDMONTON

Variety	1924–25	1925–26	1926–27	1927–28	1928–29	Average
Minhardi	60	93	70	90	95	82
Turkey	63	83	30	90	95	72
Fulcaster	15	48	10	45	25	29
Squarehead's Master	5	—	0	2	5	3

The spring wheat varieties were Marquis and Red Bobs. These, of course, are non-hardy. They were included in these studies in order to ascertain if there were characteristic differences in the low-temperature respiration of spring and winter wheats and to provide additional non-hardy material.

Apparatus

Since the rates of respiration of plants are most readily compared by measuring the rate of evolution of carbon dioxide, the devising of an apparatus for this purpose was the first consideration. The small amount of carbon dioxide evolved and the unusual temperature conditions raised numerous technical difficulties. These have been overcome and it is believed that the apparatus and method here described are satisfactory for the investigation.

Constant low temperatures are maintained by carrying out the determination in a refrigerator cooled by means of a "Frigidaire" cooling system. By making small adjustments in the starting mechanism of the "Frigidaire" motor it has been possible to obtain temperatures constant to 0.5° C. within a range of seven degrees above and below 0° C.

Reference to the photograph (Fig. 1) will show that the apparatus is built on and in the refrigerator. A continuous current of air is blown through the respiration chambers by means of a small rotary fan pump. The air is first freed from carbon dioxide by passing through a washing tower. Four of these

are hooked up in parallel in order to increase the efficiency of washing by cutting down the rate of bubbling in each. The towers are of the same construction as those used to absorb the carbon dioxide produced by respiration. A glass tube of 60 cm. length and 2.5 cm. diameter, constricted at



FIG. 1. *Apparatus used in low temperature respiration studies.*

the base so as to hold a perforated Gooch crucible plate, is tightly fitted through a rubber collar into a 300 cc. Erlenmeyer side-necked flask, the tube touching the bottom. The tube is filled to a height of 35 cm. with perforated glass beads, and 50 cc. of 20% sodium hydroxide solution is run in. A rubber stopper at the top of the tower carries the outlet tube. The lead from the washing towers enters a small bottle which acts as a trap for any sodium hydroxide which may froth over. The air is then conducted by means of a thick-walled rubber tube through a small hole in the refrigerator and into a large vertical brass tube, 70 cm. long and 2.5 cm. in diameter, the base of which is fitted into a small bottle by means of a rubber stopper. This tube collects the moisture deposited by the air on entering the refrigerator. The large diameter and upright position prevent stoppage of the air passage by the ice collected, which has been found to occur if the smaller tubes of the cooling system are connected directly with the washing towers. The air is next passed through 20 ft. of copper tubing, the coils of which are situated close to the cooling apparatus. It is then split into five currents, one entering the top of each of five respiration chambers. A thick-walled rubber tube conducts the air from the bottom of each respiration chamber through the top of the refrigerator to each of five absorption towers containing 50 cc. of 0.3 *N* sodium hydroxide solution. The rates of circulation of air through the towers are measured by capillary flowmeters. The air from each tower passes into a 500 cc. Erlenmeyer flask, which acts as an air-cushion and steadies the pressure, then through the flowmeter and out of the apparatus. The rate of flow through each respiration chamber is regulated to 15 litres per hour by means of a stopcock in the tube connecting the top of the tower and the flask.

The respiration chambers are galvanized iron cylinders of $3\frac{1}{2}$ litres capacity, closed at the top and with inlet and outlet tubes at the top and bottom respectively. They are sealed by means of ordinary plasticine into circular grooves, $\frac{1}{2}$ in. deep by $\frac{1}{2}$ in. wide, in a wooden block impregnated with wax.

A fan keeps the temperature uniform throughout the refrigerator, and temperature records are kept by means of a recording thermometer and an alcohol thermometer passed through the wall of the refrigerator so that it can be read without opening the door.

Chief among the difficulties encountered in developing apparatus suitable for the measurement of very slow rates of respiration was that of washing the intake air so free of carbon dioxide that the blank might not be disproportionately large. This was effected as described by the use of four bead washing towers containing 20% sodium hydroxide solution and hooked up in parallel. The use of a washing system of such high resistance was made possible by a pumping rather than a suction system of air circulation. After the air had passed through the washing towers, the residual positive pressure in the respiration chambers was only about 3 cm. of mercury. This had the further advantage of making it impossible for any air to leak into the apparatus. The air-washing system is not perfect but was the most practical and efficient of a number which were tried. Blank runs always showed a small amount of carbon dioxide. This, however, was fairly constant and introduced no

appreciable error. In Tables II and III are given the blank determinations for all the runs in 1927 and 1928, of which the respiration results are recorded in Tables V to X. If reference is made to the latter tables, it must be remembered that the respiration rates are expressed as mg. carbon dioxide per gm. dry matter in the plants. The blanks were subtracted from the total carbon dioxide produced by all the plants in a given chamber, and were not excessive in relation to the whole amount so produced.

TABLE II

BLANK DETERMINATIONS, 1927

Date	CO_2 mg.	Date	CO_2 mg.	Date	CO_2 mg.	Date	CO_2 mg.
Oct. 23	2.6	Oct. 26	2.3	Oct. 24	3.6	Nov. 1	2.4
Oct. 23	2.7	Oct. 26	2.5	Oct. 25	2.7	Nov. 3	2.6
Oct. 23	2.9	Oct. 26	2.4	Oct. 27	2.5	Nov. 4	2.5
Oct. 23	2.9	Oct. 26	2.7	Oct. 28	2.2	Nov. 10	2.4
Oct. 23	2.6	Oct. 26	2.5	Oct. 31	2.5	Nov. 11	2.5

TABLE III

BLANK DETERMINATIONS, 1928

Date	CO_2 mg.	Date	CO_2 mg.	Date	CO_2 mg.	Date	CO_2 mg.
Sept. 10	3.2	Sept. 27	2.4	Oct. 17	4.2	Nov. 5	2.1*
Sept. 11	4.0	Sept. 28	1.7*	Oct. 18	3.4*	Nov. 6	3.8
Sept. 12	3.3*	Sept. 29	2.0	Oct. 19	3.6	Nov. 7	4.5
Sept. 13	2.3	Oct. 1	2.2	Oct. 22	4.1	Nov. 8	4.8
Sept. 14	2.8*	Oct. 2	3.8	Oct. 23	4.8	Nov. 9	3.2*
Sept. 15	2.4	Oct. 3	4.9	Oct. 24	2.4*	Nov. 10	4.7
Sept. 16	2.4	Oct. 8	2.9*	Oct. 25	2.2	Nov. 17	5.1*
Sept. 17	2.7	Oct. 9	3.1	Oct. 26	5.3	Nov. 20	5.0
Sept. 18	2.6	Oct. 10	3.5	Oct. 29	4.3	Nov. 21	0.5*
Sept. 19	4.2	Oct. 11	4.2	Oct. 30	4.3	Nov. 23	1.7
Sept. 20	2.7*	Oct. 12	3.5*	Oct. 31	2.5*	Nov. 27	1.7
Sept. 24	2.4	Oct. 13	3.5	Nov. 1	2.2	Nov. 28	2.4
Sept. 25	1.5*	Oct. 15	3.5	Nov. 2	1.9	Nov. 30	3.8
Sept. 26	1.9	Oct. 16	4.2	Nov. 3	4.2		

**Air-washing towers filled with fresh 20% solution of NaOH.*

An interesting point was noticed in the efficiency history of the washing towers in 1928. The figures marked with an asterisk in Table III represent the blanks found in the first run after changing the absorbing solution. In twelve out of fourteen cases, the fresh solution reduced the size of the blank. In one case six runs were made before changing the solution; in all others, five or less. The apparent falling off in absorbing efficiency is not easily explained, since calculation shows that the reduction in concentration of the solution is comparatively slight, but it indicates the importance of frequent changes.

Before adopting bead towers for absorbing the carbon dioxide produced in respiration, they were tested in comparison with Reiset towers. The former

are equally as efficient as the latter and have these advantages: that they required only one third as much reagent, an advantage in titration; that they can be much more easily cleaned, and that they are much cheaper. Sodium hydroxide is used in preference to barium hydroxide as the absence of precipitate makes the rinsing of the towers more efficient, and because it allows a simpler method of titration.

The foregoing description is of the final apparatus used in obtaining all results reported in this paper. Four chambers were used for plants in each series and the fifth remained empty for the blank determination.

Procedure

Before going out to the field to make collections the apparatus is hooked up completely, except for the respiration chambers, by joining the five-way joint to the tubes leading to the absorption towers. Fifty cc. of water are run into each tower and after starting the pump the rates of bubbling are adjusted by means of the stopcocks at the top. This preliminary run clears the apparatus of residual carbon dioxide. The windows and the outside door of the room are opened in order to allow it to reach a temperature as near that of the open as possible.

In making collections, loose snow, if present, is first carefully brushed off, and then if the ground is frozen a small axe is used to cut out lumps of earth containing the plants. The plants are brought back to the laboratory and the work of preparing them for the respiration chambers is carried out on a table outside the building. Dead or wilted leaves are stripped and dead leaf-tips cut off. The earth is carefully broken away from each plant and the roots are cut off just below the crown which is wrapped in a small ball of plasticine by means of which the plant is fastened to the base of the respiration chamber. About twelve to twenty-five plants, the number depending on their size, are thus grouped together. The covering chamber is then firmly pressed into the plasticine seal. The fan is stopped and the respiration chambers are carried in and hooked up as quickly as possible. The refrigerator is open about three minutes during this operation and the temperature rises slightly. After starting the fan and pump and readjusting the rates of bubbling, the temperature is brought down to normal by running the refrigerating machine continuously.

The apparatus is now given a further run of 2 hr. in order to bring the concentration of carbon dioxide throughout the system to equilibrium. The pump is then turned off and the absorption towers are cut off from the respiration chambers by means of screw clamps. The towers are disconnected and the water is run out through the side-neck of the flask. They are replaced and 50 cc. of 0.3 N sodium hydroxide is run into each from a standard pipette suitably protected from contamination by the carbon dioxide of the air. The actual determination of carbon dioxide evolved is now made by running the apparatus for 4 hr. The pump is then turned off and the stopcocks at the top and the screw clamps at the bottom of the towers are closed.

The titration is carried out in the original flask. The tube is drawn up from the bottom of the flask, washed down with eight small quantities of carbon-dioxide-free water, and the volume is then made up to 200 cc. Two drops of phenolphthalein are added, and the solution is titrated with approximately 0.3 *N* hydrochloric acid until the color begins to pale. The titration is then continued with 0.05 *N* standard hydrochloric acid until the colorless end-point is reached. Three drops of the British Drug House Universal Indicator are added and the titration continued until a slight change in the quality of the color gives the first true pink. The amount of carbon dioxide absorbed is calculated from the amount of acid required to titrate from the first, or bicarbonate end-point, to the final carbonate end-point. The above technique was adopted after experimenting with various combinations and quantities of different indicators, as being the most accurate and convenient for the determination of the amounts of carbonate usually present. Tests carried out with known solutions of sodium bicarbonate gave a recovery of 100% with a maximum error of 1%.

Experiments of 1927

The results prior to Oct. 24, 1927, are not reported, because, as already mentioned, the apparatus and procedure were being continually improved and it is felt that after these had taken final form a sufficient body of reliable data was obtained. The earlier data, though less precise than those secured later, support the same general conclusions.

The results of the respiration experiments extending from Oct. 24 to Nov. 11, 1927, are given in Tables V and VI, reporting respectively, a series with Minhardi to check the error of sampling, and a series with the four winter varieties.

In Table IV are given the minimum and maximum daily temperatures during and shortly before the period of the 1927 experiments. The columns here and in other tables of temperature are given in the reverse order to that usually

TABLE IV

TEMPERATURE RECORDS (°F.) DURING PERIOD OF RESPIRATION EXPERIMENTS, 1927

Date	Min.	Max.	Date	Min.	Max.	Date	Min.	Max.	Date	Min.	Max.
Oct. 19	34	55	Oct. 26	24	50	Nov. 2	12	51	Nov. 9	10	20
20	27	49	27	18	44	3	33	42	10	5	9
21	26	57	28	31	47	4	21	40	11	−7	8
22	29	55	29	31	48	5	13	39	12	−14	11
23	27	56	30	15	41	6	17	21	13	−14	6
24	23	51	31	18	33	7	10	19	14	−14	7
25	26	51	Nov. 1	12	37	8	10	26	15	− 4	9

found in meteorological records, to have the readings in chronological order; *i.e.*, the minimum temperature for a given day usually occurs early in the morning.

After the apparatus had been brought to a state of maximum efficiency, the first experiment was designed to test the error of sampling. In Table V are given the results of three runs with Minhardi, made from Oct. 24 to 27, a period of very even temperatures (Table IV) except for a colder dip on the morning of the 27th, which may perhaps account for the slightly lower average rate of respiration on that day. For any one run, however, it seems legitimate to attribute the variations between replicates chiefly to sampling errors. Twelve plants were used in each chamber, and while an effort was made to select only plants which appeared representative, it is not surprising to find this small number insufficient to get rid of the natural variability of such material. This emphasized the importance of making as many runs as possible on which to base comparisons of varieties.

TABLE V

RESPIRATION MEASUREMENTS OF REPLICATE SAMPLES OF MINHARDI WHEAT AT 0° C.

Sample No.*	Mg. CO_2 per gm. dry matter per 4 hr. run			
	Oct. 24	Oct. 25	Oct. 27	Average
1.	1.67	1.67	1.77	1.70
2.	1.82	1.95	1.65	1.81
3.	1.96	1.96	1.75	1.89
4.	1.88	1.83	1.63	1.78
Average	1.83	1.85	1.70	1.79

**Samples collected at 11 a.m.*

From October 6 to October 24, when the foregoing special experiment with Minhardi was done, the weather had become gradually colder. On October 7 the temperature fell to 18° F. This killed back the tips of the leaves of the two spring varieties to such an extent that they were useless for further work. The series of runs reported in Table VI was thus restricted to the winter varieties.

TABLE VI

RESPIRATION MEASUREMENTS OF WINTER WHEATS AT −7°, 0° AND 7° C., OCT. 28–NOV. 11, 1927

Variety*	Mg. carbon dioxide per gm. dry matter per 4 hr. run									
	−7° C.			0° C.				7° C.		
	Nov.4	Nov.10	Av.	Oct.28	Oct. 31	Nov.1	Av.	Nov.3	Nov.11	Av.
Minhardi	0.56	0.46	0.51	1.58	1.76	1.78	1.77	2.96	4.44	3.70
Turkey	0.49	0.42	0.46	1.66	1.60	1.68	1.65	2.60	3.77	3.19
Fulcaster	0.86	0.50	0.68	1.45	1.67	1.88	1.67	3.07	4.78	3.93
Sq. Master	0.86	0.53	0.70	1.63	1.88	2.09	1.87	3.30	3.68	3.49

**Samples collected at 11 a.m.*

which by now had been hardened by exposure to fairly severe frosts. The plants were still comparatively undamaged, although the oldest leaves had to be removed, because brown and dead, and the tips of some of the other leaves cut off, because killed by frost. The cold snap beginning on the night of Nov. 9-10, however, killed a considerable number of the leaves of Fulcaster and Squarehead's Master, and the results obtained with these varieties on Nov. 10 and 11 must therefore be accepted with reserve.

It will be noted that in Table VI and all other tables of respiration results, the varieties are arranged in order of winter hardiness.

Considering all the results in Table VI, it appears that Fulcaster and Squarehead's Master, the two non-hardy varieties, respired more rapidly than the others at −7° C., that Squarehead's Master had the greatest rate at 0° C., and that at 7° C. there were no definite differences. If we eliminate the runs of Nov. 10 and 11, when Fulcaster and Squarehead's Master had been injured by frost, the differences at −7° C. are increased; these two varieties also respired slightly more rapidly than the others at 7° C. Bearing in mind the variation between replicates, not much weight should be given to the differences found at temperatures other than −7° C., but the results on the whole are in agreement with those found earlier in the same season as well as in the previous seasons.

On November 14 all varieties were collected, thawed out gradually and examined. Almost all the leaves of Squarehead's Master were dark green and watery looking. Fulcaster was not much better and 80% of the leaves of Turkey were in a similar condition. In Minhardi only the tips of the leaves

TABLE VII

TEMPERATURE RECORDS (°F.) DURING PERIOD OF RESPIRATION EXPERIMENTS, 1928

Date	Min.	Max.	Date	Min.	Max.	Date	Min.	Max.	Date	Min.	Max.
Sept.			Sept.			Oct.			Nov.		
8	28	64	29	42	80	20	23	45	10	22	42
9	32	71	30	39	52	21	18	56	11	17	46
10	33	75	Oct.			22	30	55	12	23	53
11	37	71	1	33	54	23	26	50	13	15	38
12	40	73	2	36	43	24	17	52	14	15	33
13	37	75	3	28	40	25	25	56	15	8	37
14	47	65	4	17	41	26	28	37	16	8	37
15	37	60	5	22	49	27	18	33	17	17	37
16	27	69	6	25	61	28	17	40	18	5	37
17	35	80	7	30	56	29	13	40	19	13	43
18	37	67	8	32	52	30	11	33	20	14	43
19	34	53	9	29	52	31	8	29	21	25	42
20	38	62	10	33	38	Nov.			22	19	52
21	44	65	11	21	39	1	8	42	23	20	38
22	43	57	12	20	44	2	9	55	24	10	42
23	41	55	13	23	59	3	10	58	25	14	43
24	25	57	14	28	58	4	14	52	26	9	34
25	21	55	15	24	55	5	20	48	27	10	22
26	18	62	16	26	59	6	20	50	28	10	37
27	22	65	17	35	40	7	18	48	29	10	38
28	32	80	18	25	55	8	16	49	30	21	39
			19	29	52	9	18	52			

were damaged. The plants were afterwards put in water and kept at about 12° C. The dark green leaves failed to become turgid and proved to be dead. Under these conditions it was considered useless to continue the investigation. The work of 1927 and of the preceding two years, however, besides providing useful indications of the relations between varieties, resulted we believe in the development of the maximum efficiency possible in measuring respiration rates with the type of apparatus used. More reliable results can only be obtained by growing the plants under controlled conditions.

Experiments of 1928

It has already been stated that the field plots in 1928 were covered with burlap every night after Sept. 28, to protect the plants from snow and also to some extent from frost-killing of the leaf-tips. The object was to prolong the period during which respiration measurements could be made with uninjured plants. In order to lengthen the season further by starting the experiments earlier, two seedings were made of all varieties, the first on Aug. 3 and the second on Aug. 20. The latter is the normal date for seeding winter wheat at Edmonton. The temperature records for the period of the experiments are given in Table VII. The plants for all experiments this season were collected at about 10 a.m. on the dates specified in the tables.

By the time the respiration determinations were started on September 10 the spring wheat plants of the first seeding (Aug. 3) had grown to a height of 12 in. The respiration chambers used were only 7½ in. high so that although the number of plants per run was reduced, they had to be crowded and perhaps slightly crushed in order to get them into the chambers. The winter wheats, of course, had not made so much growth, the average height of the plants being about 6 in. These plants were quite satisfactory in size.

After September 20, plants seeded on August 20 were used. These were within the limitations of size imposed by the design of the respiration chambers.

From September 24 to 27 there were heavy frosts each night. Considerable damage was done to the spring wheats but none to the winter varieties. By September 29 half the leaves of Marquis and Red Bobs had to be stripped entirely and the tips of many of the remaining leaves cut back, so that only sound tissue might be used for the determinations. Up to the 10th of October the damage to the spring wheats became progressively worse. By that date only one or two green leaves remained on each plant and the work with these varieties was discontinued. During this period the winter wheats remained in good condition.

By October 17 Squarehead's Master began to show injury at the leaf tips and by the 25th many of the leaves had to be removed entirely and most of the rest showed tip injury. During the nights of October 29-31 heavy frosts touched the leaf-tips of Turkey and Fulcaster. The Minhardi plants still remained normal.

No further heavy frosts came until the week of Nov. 10-17. After these frosts the Minhardi showed injury to the leaf tips. The three hardier varieties (Minhardi, Turkey and Fulcaster) all showed considerable wilting, a result of

prolonged exposure to dry, cold weather. The wilted leaves were stripped before the experiments. The plants of Squarehead's Master had only one or two leaves apiece in suitable condition for the tests. The experiments were discontinued on November 30.

TABLE VIII

RESPIRATION MEASUREMENTS OF WINTER AND SPRING WHEATS AT −7°, 0° AND 7° C., 1928

Variety	Mg. carbon dioxide per gm. dry matter per 4 hr. run													
	−7°C.				0°C.				7°C.					
(a)	Sept. 17	Sept. 18	Sept. 19	Sept. 20	Sept. 10	Sept. 11	Sept. 12	Sept. 16	Sept. 13	Sept. 14	Sept. 15			
Minhardi	1.50	1.26	—	1.05	2.15	—	2.26	—	4.86	—	3.10			
Turkey	—	—	0.92	1.23	—	1.88	2.08	2.02	—	4.71	5.12			
Fulcaster	—	1.34	1.25	—	2.08	—	—	1.56	4.18	4.19	—			
Sq. Master	1.85	1.63	—	—	2.44	1.97	—	2.13	5.14	5.25	—			
Red Bobs	—	—	1.38	—	—	1.98	1.93	—	—	4.07	4.55			
Marquis	1.54	—	—	1.37	2.56	—	2.11	1.74	4.09	—	4.64			
	−7°C.						0°C.			7°C.				
(b)	Oct. 1	Oct. 2	Oct. 3	Oct. 8	Oct. 9	Oct. 10	Sept. 24	Sept. 25	Sept. 26	Sept. 27	Sept. 28	Sept. 29		
Minhardi	0.86	—	—	0.76	1.06	—	2.29	—	2.38	4.30	—	4.55		
Turkey	0.98	—	0.81	1.05	1.27	—	—	2.18	2.40	—	4.25	4.24		
Fulcaster	—	1.14	0.85	1.14	—	1.22	1.73	2.17	—	4.90	4.41	—		
Sq. Master	—	1.18	0.87	0.91	—	1.52	2.62	2.66	—	4.47	3.68	—		
Red Bobs	1.08	—	0.79	—	1.53	1.69	—	2.61	2.37	—	3.97	4.04		
Marquis	1.27	1.63	—	—	1.82	1.85	2.24	—	2.36	3.98	—	5.03		
	−7°C.					0°C.				7°C.				
(c)	Oct. 12	Oct. 16	Oct. 19	Oct. 24	Oct. 26	Oct. 11	Oct. 15	Oct. 18	Oct. 23	Oct. 29	Oct. 13	Oct. 17	Oct. 22	Oct. 25
Minhardi	0.67	0.78	0.23	0.89	0.70	1.69	1.52	1.59	1.34	1.63	4.25	2.98	2.96	2.96
Turkey	0.71	0.91	0.49	0.87	0.75	1.45	1.60	1.64	1.19	1.76	4.38	3.02	3.20	3.35
Fulcaster	0.90	1.09	0.84	0.93	0.83	1.64	1.20	1.41	1.55	1.87	4.45	3.04	3.00	3.05
Sq. Master	1.07	1.14	0.93	1.20	0.80	1.98	1.53	1.94	1.76	2.39	5.77	4.64	3.87	3.70
	−7°C.				0°C.			7°C.						
(d)	Oct. 31	Nov. 2	Nov. 7	Nov. 9	Nov. 1	Nov. 5	Nov. 8	Oct. 30	Nov. 3	Nov. 6	Nov. 10			
Minhardi	0.35	0.78	0.70	0.61	1.20	1.47	1.18	3.33	2.71	2.51	2.39			
Turkey	0.54	—	0.65	0.73	1.43	1.53	0.94	3.31	2.96	2.21	2.70			
Fulcaster	0.60	1.12	0.88	0.89	1.45	1.37	1.32	3.78	3.06	2.55	2.59			
Sq. Master	0.81	1.23	0.81	0.85	1.31	1.77	1.83	4.49	3.77	2.35	3.58			
	−7°C.			0°C.			7°C.							
(e)	Nov. 21	Nov. 30	Nov. 17	Nov. 20	Nov. 27	Nov. 23	Nov. 28							
Minhardi	0.43	0.60	1.07	1.28	0.94	2.44	2.64							
Turkey	0.72	0.39	1.30	1.16	1.04	1.90	2.90							
Fulcaster	0.86	0.36	—	1.43	1.07	2.88	2.17							
Sq. Master	0.67	0.55	—	1.66	1.60	3.11	2.62							

The results of the 55 runs made in 1928 are in Table VIII divided into five sections, corresponding to the periods described above, *viz.*,: (a) while plants from the first seeding were used; (b) while it was possible to continue the experiments with both winter and spring wheats from the second seeding; (c) after the spring wheats had been discontinued because of frost damage, and while all the winter wheats but Squarehead's Master remained untouched by frost; (d) after the leaf-tips of Turkey and Fulcaster had been touched; (e) after Minhardi leaf-tips showed injury. Since only four varieties could be included in one run, a rotation was necessary when both spring and winter varieties were used. Accidents sometimes reduced the number of records obtained to three, especially in the first section of the table. After the spring varieties were discontinued, all the others were included in every run, and the three experimental temperatures were used in rotation, generally on successive days.

The results show considerable variation in the respiration of samples of the same variety at the same temperature on different days. No doubt this is due partly to the natural variability of the plants, an unavoidable source of error already noted, and partly to changes in weather conditions. The general effect of temperature is clearly seen in the gradual reduction of respiration rates as the season advances. To minimize the effect of sampling errors and temperature fluctuations, the data have been summarized in Table IX by periods corresponding to the sections of Table VIII. Each figure is the average rate found in all the runs of a given period.

TABLE IX

SUMMARY OF RESPIRATION MEASUREMENTS, 1928

Variety	Mg. CO_2 per gm. dry matter per 4-hr. run			Variety	Mg. CO_2 per gm. dry matter per 4-hr. run		
	−7°C.	0°C.	7°C.		−7°C.	0°C.	7°C.
(a) Sept. 10–20				(c) Oct. 11–29			
Minhardi	1.27	2.20	3.98	Minhardi	0.65	1.59	3.19
Turkey	1.08	1.99	4.92	Turkey	0.75	1.52	3.49
Fulcaster	1.30	1.82	4.18	Fulcaster	0.92	1.53	3.38
Sq. Master	1.74	2.18	5.20	Sq. Master	1.03	1.92	4.50
Red Bobs	1.38	1.96	4.31	(d) Oct. 30–Nov. 10			
Marquis	1.46	2.14	4.36	Minhardi	0.61	1.28	2.74
(b) Sept. 24–Oct. 10				Turkey	0.64	1.27	2.80
Minhardi	0.89	2.34	4.42	Fulcaster	0.87	1.35	3.00
Turkey	1.03	2.29	4.24	Sq. Master	0.92	1.64	3.55
Fulcaster	1.09	1.95	4.66	(e) Nov. 17–30			
Sq. Master	1.12	2.64	4.08	Minhardi	0.51	1.10	2.54
Red Bobs	1.27	2.49	4.00	Turkey	0.55	1.17	2.40
Marquis	1.64	2.30	4.50	Fulcaster	0.61	1.25	2.52
				Sq. Master	0.61	1.63	2.86

As already noted, the spring wheat plants used during the first period were seeded on Aug. 3, and were so large they had to be crowded into the respiration chambers. This crowding may on the one hand have restricted the free circulation of air and so cut down the rate of respiration, while on the other hand

enough tissue may have been crushed to accelerate the rate of evolution of carbon dioxide. No net effect of the crowding is shown by the results, which at 0° and 7° C. were not significantly different from those obtained with winter wheat plants of smaller size. As all varieties were during this period in an unhardened condition, differences related to winter hardiness would scarcely be expected. There is, however, an indication that the spring wheats and Squarehead's Master, an English winter wheat definitely non-hardy at Edmonton, respired slightly faster than the others at −7° C.

The change on September 24 to the smaller plants seeded on August 20 coincided with the incidence of heavier frosts. The period therefore includes all determinations made with spring wheat plants from which gradually increasing amounts of tissue had to be discarded because of frost damage, and winter wheats in a partly hardened but undamaged condition. Here some additional evidence of differentiation appears in the respiration rates at −7° C., which fall in the inverse order of hardiness of the winter wheats, with the spring wheats definitely faster than the least hardy winter variety. At 0° and 7° C., no differences were demonstrated.

The remaining three periods of experiments with winter wheats are characterized by gradually decreasing rates of respiration and a sharper differentiation on the basis of hardiness, which now becomes evident at 0° C. though still more distinct at −7° C.

The results of the entire series of 1928 experiments will now be analyzed in greater detail, with special reference to the winter varieties.

Measurements at −7° C. In Table VIII it will be seen that there were ten runs at −7° C. in which all four winter varieties were compared, and eleven additional runs in which two or three of them were included. In fourteen of these twenty-one runs the values for all varieties fell in the inverse order of hardiness. In Table X are shown the results obtained in the remaining seven runs. It will be seen that in four out of the seven runs the two hardy varieties fall in one group characterized by slower respiration than the other group of two non-hardy varieties. The difference in hardiness between Minhardi and Turkey, or between Fulcaster and Squarehead's Master, is not as great as the difference between the pairs. The latter difference is so great that the application of the terms hardy and non-hardy respectively is amply justified. The division of the results into two groups is therefore almost as satisfactory as if they had come in the expected order for individual varieties. Two of the three series of measurements given in Table X which show no regularity are the last two of the season and were made at a time when the plants of all varieties were showing considerable frost injury. Including all runs, however, there are out of the twenty-one just three which do not support the conclusion that hardy varieties have a lower rate of respiration than non-hardy varieties at −7° C.

Measurements at 0° C. The summaries of the measurements made at 0° C. (Table IX) show no regularity until the last two periods. From Oct. 30 to Nov. 10, Minhardi and Turkey had the same average rate of respiration, and the two non-hardy varieties higher rates, the least hardy being highest. In the last period the rate of respiration was in all varieties inversely proportional to

TABLE X

RESULTS OF THOSE MEASUREMENTS AT −7°C. WHICH DID NOT FALL IN INVERSE ORDER OF HARDINESS FOR ALL VARIETIES

Date 1928	Mg. carbon dioxide per gm. dry matter per 4 hr. run			
	Hardy varieties		Non-hardy varieties	
	Minhardi	Turkey	Fulcaster	Sq. Master
Oct. 8	0.76	1.05	1.14	0.91
Oct. 24*	0.89	0.87	0.93	1.20
Oct. 26*	0.70	0.75	0.83	0.80
Nov. 7*	0.70	0.65	0.88	0.81
Nov. 9*	0.61	0.73	0.89	0.85
Nov. 21	0.43	0.72	0.86	0.67
Nov. 30	0.60	0.39	0.36	0.55

**In these series the pairs of hardy and non-hardy varieties did fall in inverse order of hardiness.*

hardiness, as at −7° C. A similar condition is of course shown by the data for the individual runs in Table VIII. These show no regularity until October 23. Of the seven runs made after that date, the results of three fall in the expected order for all varieties, three divide the varieties into the hardy and non-hardy groups, and in the one remaining run Fulcaster had the lowest rate of respiration. This is apparently fairly typical of the behavior of this variety at this temperature. In the second and third periods the results for Fulcaster are lower than its hardiness would lead one to expect.

Measurements at 7° C. The measurements at 7° C. show little definite trend at any stage of the experiment. Only in three of the sixteen runs did the results for all four varieties fall in the inverse order of winter hardiness, though in a few other cases they fell into the hardy and non-hardy pairs. Whether these cases are fortuitous or significant it is difficult to say.

Discussion

The results presented after four seasons study of the respiration of wheat plants at low temperature are considered to establish definitely that at −7° C. the rate in winter varieties is in the inverse order of hardiness. The differentiation is indefinite early in the season, but becomes increasingly sharp as the plants advance into the fully hardened condition. At 0° C. the same differences could be observed, in a less marked degree, after the plants had become hardened. At 7° C. the relationship practically disappeared.

During the time in which it was possible to work with the spring wheats, they showed, as compared with the winter varieties, a higher rate of respiration at −7° C. Their susceptibility to frost made it impracticable to determine whether this differentiation would extend to the rate at 0° C. in the hardened condition. The results as they stand may be regarded as confirming and extending the conclusion that at −7° C. the rate of respiration in wheat plants is inversely proportional to winter hardiness.

The significance of this conclusion lies in the fact that in this climate winter plants are for long periods covered with snow at temperatures below the freezing point. Under these conditions the hardier varieties of wheat would appear to conserve more effectively their sugar reserves, which are an important protection against frost denaturation of the cell proteins. Furthermore, the slower respiration at freezing temperatures is an expression of the plant's ability to go more rapidly and completely into a dormant condition in which sensitivity to frost injury is reduced. The latter change is probably connected with the colloidal condition of the cell contents.

The decrease in rate of respiration as the season progresses is probably a temperature effect and one manifestation of the hardening process. All varieties started from approximately the same level, but the reduction in rate was carried farther in the hardy varieties.

Earlier investigations in this laboratory have shown the leaf blades to be considerably richer in sugar than the leaf sheaths. It might therefore be suggested that the cutting-back or removal of leaf blades in the later periods of the respiration experiments would reduce the net concentration of sugar in the tissues and so depress the rate of respiration. To answer this, it may be pointed out that sugar concentration is evidently not the limiting factor, since hardy varieties contain more sugar and yet respire more slowly. Moreover, Minhardi, of which the leaves remained untouched until the last period in 1928, fell off in respiration rate more markedly than any of the others. On the other hand, it may be argued that the cutting and bruising of the tissues incident to the removal of blades and tips would be expected to accelerate respiratory reactions. Following the heavy frosts of the nights of October 29-31, 1928, which touched the leaf-tips of Turkey and Fulcaster, the difference between the rates of respiration of these varieties and Minhardi, which remained untouched, were accentuated. Against this, however, must be set the observation of a similar differentiation between the winter varieties after the first hardening frosts of October and before any injury to the tissues had taken place. On the whole, therefore, it seems legitimate to regard the changes as an expression of the relative adaptations of the varieties to winter resistance.

A comparison of the daily variation in respiratory rates with the daily fluctuations in outdoor temperature suggests that there is a considerable lag in the response of physiological state to temperature. The comparative stability of the progressive change in state of the plants brought about by the hardening process is clearly shown in the respiration rates found, which decreased throughout the season, notwithstanding that all measurements were made at the same series of temperatures. On the other hand, physiological activity, within the limits of the state of dormancy at any given time, evidently responds very rapidly to temperature, since only 2 hr. were allowed for plants to come to equilibrium in the refrigerator before making the measurement, and at 7° C. all varieties respired at about the same rate, whereas at −7° C. they were sharply differentiated.

Of the two suggestions put forward at the outset of this work, to account for the slower rate of sugar loss from hardy varieties, only the first, that they might have a more profound dormancy characterized by a slower rate of respiration at winter temperatures, has been proved by these experiments. The other suggestion, that they might have a more stable dormancy and be less easily wakened into a more frost-susceptible condition of physiological activity by occasional warm periods during the winter, seems not to be supported by the behavior of the varieties at 7° C. It has been noted, however, that these were only small changes in activity within the limits of the state of dormancy at the time. The possible effect on this state of a thaw lasting some days has still to be investigated.

In passing, it may be noted that catalase activity, which is believed by some investigators to be directly related to respiratory activity, was found to be directly related to winter hardiness in wheat (5). Though the leaves of hardened plants were used, the catalase determinations on the press-juice were carried out at 20° C. The present experiments do not make it possible to say whether the respiratory activity of hardened plants at this temperature would be in the order of hardiness. It is clear, however, that catalase activity as ordinarily determined is inversely related to low-temperature respiration.

References

1. Åkerman, Å. Studien über den Kältetod und die Kälteresistenz der Pflanzen nebst Untersuchungen über die Winterfestigkeit des Weizens. Lund, 1927.
2. Govorov, L. I. The diverse characters of winter and spring forms of cereals in connection with the problem of hardiness in winter crops. Bull. Appl. Bot. and Plant Breeding, 13: 525-559. (Russ. with Engl. abs.). 1923.
3. Martin, J. H. Comparative studies of winter hardiness in wheat. J. Agr. Research, 35: 493-535. 1927.
4. Newton, R. and Brown, W. R. Frost precipitation of proteins of plant juice. Can. J. Research, 5: 87-110. 1931.
5. Newton, R. and Brown, W. R. Catalase activity of wheat leaf juice in relation to frost resistance. Can. J. Research, 5:333-336. 1931.
6. Newton, R., Brown, W. R. and Anderson, J. A. Chemical changes in nitrogen fractions of plant juice on exposure to frost. Can. J. Research, 5:327-332. 1931.

PREPARATION OF GLUTENIN IN UREA SOLUTIONS[1]

By W. H. Cook[2] and C. L. Alsberg[3]

Abstract

A new method has been developed for preparing glutenin, using concentrated urea solution as a dispersion medium. The starch is removed from the dispersion by passing it through a Sharples supercentrifuge. The glutenin is then removed from the gliadin by precipitation: (a) by adding magnesium sulphate to about 0.17 of saturation; or (b) by adding water until the urea is diluted to about 10% concentration. Alcohol precipitation is unsatisfactory, since the glutenin loses much of its original solubility. Drying, even at room temperature, renders glutenin insoluble in 30% urea solution. Different samples of glutenin isolated by the urea method have similar amide and arginine nitrogen contents. The amount of these constituents is intermediate between the values reported for glutenin isolated from alkali, and that isolated from acid. While urea solutions denature some proteins, they affect glutenin less than dilute alkalies, as judged by the sulphydryl test, and no more than dilute acids. Neutral urea solutions permit a study of the physical properties of glutenin without previous exposure to extremes of hydrogen ion concentration. This method should be applicable to the isolation of glutenins from other seeds.

Introduction

Glutenin is described throughout the literature as being insoluble in neutral solutions. It is consequently not surprising to find that the methods described for its isolation involve a temporary dispersion in acid or alkali. Ordinarily they also include an extraction with alcohol, to remove the gliadin with which it is associated. Since the above reagents are known to denature other proteins it seems probable that glutenin, isolated by the customary methods, may have undergone changes which significantly alter its physical properties, if not its chemical composition. Since the physical properties of glutenin are regarded as of primary importance in determining the baking quality of flour, an attempt has been made in the present investigation to find a neutral dispersion medium which could be employed for the preparation and subsequent study of glutenin in its normal isoelectric state. Concentrated solutions of urea appear to fulfil the necessary conditions.

Protein Denaturation

A decrease in solubility is the first indication that a protein is altered by a given treatment. The generally accepted view as to the nature of this process is that of Hardy (20) and Chick and Martin (7) who conclude that it takes place in two stages. The first of these is termed denaturation, and although its exact nature is obscure, it is regarded as a change in the chemical structure of the protein molecule. It is not yet known whether the various forms of denaturation represent identical changes. The second stage is the flocculation

[1] *Contribution from the Food Research Institute and Department of Chemistry, Stanford University, California, and the Department of Field Crops, University of Alberta, with financial assistance from the National Research Council of Canada. The present paper is being issued as Paper No. 25 of the Associate Committee on Grain Research. It constitutes part of a thesis presented by the senior author to the Department of Chemistry, Stanford University, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.*

[2] *Junior Research Biologist, National Research Laboratories of Canada.*

[3] *Director, Food Research Institute, Stanford University, California.*

of the denatured protein particles. This process is a general colloidal phenomenon, which proceeds most rapidly at the isoelectric point and in the presence of electrolytes.

The amount of protein which becomes insoluble, under conditions which favor flocculation, is generally taken as a measure of the denaturation resulting from a given treatment. Glutenin has not been considered amenable to study in this manner since it has generally been regarded as insoluble in neutral solutions. The denaturing influence of dilute acids and alkalies on this protein must therefore be inferred from investigations on other proteins.

Lewis (31, 32) found that the rate of heat denaturation of egg albumin and hemoglobin was minimal when the hydrogen and hydroxyl ion concentrations were equal. He also found (33) that high salt concentrations actually depressed the velocity of denaturation, a result in agreement with the earlier work of Chick and Martin (8). Wu and Yen (49) have presented considerable evidence which shows that dilute acids and alkalies denature many proteins.

Sjögren and Svedberg (43) have studied the stability of egg albumin solutions at different hydrogen ion concentrations with an ultracentrifuge. They found that between pH 4.0 and 9.0 the protein was stable and homogeneous in molecular weight, but beyond this range some of the molecules split into non-centrifugable substances. At pH values lower than 3.0 an increased sedimentation indicated the formation of aggregates of denatured protein.

The pH stability region of keratin has been investigated by Speakman and Hirst (44). They measured the resistance of wool to extension in different media, and conclude that it is completely stable from pH 4.0 to at least pH 7.0. The stability of both soluble and insoluble proteins appears therefore to be restricted to a limited range of hydrogen ion concentrations.

Prolonged exposure of proteins to acids and alkalies may cause chemical changes of a more complicated nature than those involved in denaturation. Anson and Mirsky (2), for instance, mention that alkali, after denaturing a protein, causes further changes which make the denatured material more soluble. Wu and Yen (49) report that dilute acids and alkalies, even at room temperature, cause the liberation of non-protein substances from proteins, including some of cereal origin.

In connection with the effects of acids and alkalies on glutenin, Blish (4) has shown that Halton's (19) success in fractionating this protein by precipitation with acid was doubtless the result of a partial racemization of the protein during the course of its preparation. Not only did a loss n optical rotatory power result, but Blish observed a change in the isoelectric point.

In a recent paper Blish and Sandstedt (5) have called in question the alkali dispersion method of preparing glutenin, by showing that different concentrations of alkali yielded fractions of different chemical composition. In these preparations the diamino nitrogen of glutenin increased with the concentration of alkali used in its preparation, while the arginine nitrogen in preparations from acid was lower than those prepared from alkali. These workers believe that the so-called 'glutenin' prepared by this method is a product resulting from the action of alkali on a more complex protein substance.

Similar evidence has been observed by Knaggs and Schryver (27) working with collagen. They found that the Hausmann numbers of gelatin depended on the previous treatment of the collagen, an acid treatment favoring a low diamino nitrogen and an alkali treatment the reverse. Later (28), they found that gelatin, which had been treated with cold dilute acids or alkalies, showed an increase in the monoamino nitrogen of the filtrate from the diamino nitrogen. Knaggs (26) reports that treatment of collagen with alkali or acid, at the hydrogen ion concentration which gives maximum swelling, will result respectively in a maximum or minimum amount of diamino nitrogen on hydrolysis.

The foregoing citations by no means exhaust the literature on the effects of dilute acids and alkalies on proteins. However, they suffice to show that both the physical properties and chemical composition of proteins, including glutenin, may be altered significantly when these reagents are employed for isolating the material. It also seems probable that the colloidal properties of glutenin are altered by the alcohol treatments used to remove the gliadin, since it is well known that alcohol denatures proteins. These considerations lead to the development of a new method of preparing glutenin, using a neutral dispersion medium.

Urea Solution as a Neutral Dispersing Agent

Two types of substances were considered as possible neutral dispersing agents: (1) solutions of the lyotropic salts, such as potassium iodide and potassium thiocyanate; and (2) solutions of certain organic nitrogenous compounds, such as urea.

Regarding the former, Gortner, Hoffman and Sinclair (18) reported that about two-thirds of the protein in flour is dispersed by a normal solution of potassium iodide. Stimulated by this work, the present authors, prior to this investigation, attempted to disperse washed gluten in 30% potassium iodide. Only part of the gluten was dispersed, hence the use of lyotropic salt solutions was abandoned.

The dispersing power of urea solutions has been observed by several workers. Spiro (45) has shown that this substance raises the coagulation temperature of proteins. Ramsden (40) however, was probably the first to demonstrate that strong urea solutions had a solvent action on proteins. Fischer (14) has also shown that dilute solutions stimulate markedly the swelling of fibrin. Recently, the solvent action of urea solutions on proteins has been demonstrated by Dill and Alsberg (13) and Burk and Greenberg (6), while Foulger (15) has shown that it has a similar effect on starch. Since Alsberg and Perry (1) had observed that concentrated urea solutions would disperse gluten, it was chosen for further consideration.

The denaturing effect of concentrated urea solutions on certain proteins has also been demonstrated. Anson and Mirsky (2) and Burk and Greenberg (6) have shown that hemoglobin and egg albumin are denatured by urea. Hopkins (24) has shown that the serum proteins, as well as egg albumin, are altered by urea solutions. The nature of this alteration appears to depend somewhat on the character of the protein. Burk and Greenberg (6) found that hemo-

globin dissolved in urea solution had a molecular weight one-half of that obtained in glycerol or water solutions in which it is not denatured. They also report that the molecular weight of casein and edestin in urea solution was only one-third and one-fourth, respectively, of the values reported by Svedberg (46), who used a centrifugal method, but it is not known whether or not these proteins were denatured by the urea. However, the former investigators and Huang and Wu (25) found that egg albumin was not altered in molecular weight when denatured by urea. Hopkins (24) and Ramsden (41) have presented evidence which suggests that urea denaturation involves an alteration in the chemical constitution of the protein. These investigators found that, in addition to the loss of solubility characteristic of denaturation, the denatured protein gives a marked sulphydryl test with nitroprusside. The temperature coefficient of urea denaturation was found to be negative, but at all temperatures the denaturation rate was sufficient to complete the change within a few hours.

Doubtless the changes involved in urea denaturation would be less obscure if the nature of the dispersing action of these solutions were better understood. Starch and many proteins, irrespective of class, are dispersed by urea. Spiro (45) explained this as due to the formation of alkali proteinates, the urea acting as an alkali. Fischer (14), on the other hand, presents evidence to show that the hydrating effect of urea is not a simple alkali effect, as acids do not counteract it in any concentration. Further evidence against the alkali theory has been presented by Dill and Alsberg (13). A possible explanation of the dispersing power of urea solutions is that they are highly polar, as shown by their high dielectric constant (16, 29). Since proteins themselves possess high polarities (17, 34), a mutual solubility might be expected. If this hypothesis is correct, the splitting of hemoglobin into fractions of lower molecular weight may be regarded as a physical phenomenon. This agrees with the opinion expressed by Burk and Greenberg (6), who consider that the mildness of urea precludes the possibility of an hydrolysis of the same type caused by acids, alkalies, or enzymes. The fact that the dielectric constant and the rate of denaturation both decrease as the temperature rises may be significant in this connection.

Most of the evidence that has been presented on the denaturation of proteins by urea solutions has appeared since this investigation was undertaken. However, urea solutions still appear to be better than acid or alkali solutions for the preparation of glutenin, because they permit a study of the protein in its isoelectric state, the condition in which it possesses the properties important in bread dough. Further, there appear to be fewer progressive secondary changes in protein in urea solution than in acid or alkali solution. In this respect, Burk and Greenberg (6) have shown that the molecular weight of casein remained constant for several days at 0° C., and Dill and Alsberg (13) found that the optical rotation of gliadin in 30% urea solution remained constant for 7 days at 37° C.

None of the other neutral solvents that might be used for dispersing proteins appear to be any better than urea, and all that have been tested caused

denaturation. Anson and Mirsky (2) have demonstrated that the lyotropic salts, potassium iodide and potassium thiocyanate, caused denaturation, while Hopkins (24) has shown that other nitrogen compounds similar to urea also affect the proteins. If proteins, such as glutenin, which are not dispersed by mild neutral solvents, obtain their insolubility from their state of aggregation, and if the action of reagents which disperse them is to break up these aggregates, then, judging from the action of urea on hemoglobin, all such proteins when dispersed will be denatured. Further, the recent work of Mirsky and Anson (35) suggests that the denaturation is not an entirely irreversible process, as it was once considered. Observations on the denaturing effect of urea on glutenin, as well as its solvent properties, were made in the present investigation.

Experimental

Methods of Analysis

Hydrogen ion concentration. This determination was made with a quinhydrone electrode at 25° C. A hydrogen electrode of the bubbling type could not be used with the urea solutions as the hydrogen appears to remove carbon dioxide, causing a drift in the alkaline direction.

Moisture. This determination was made in two ways: by heating at 98° C. for 48 hr. *in vacuo*, and by drying to constant weight over phosphorus pentoxide *in vacuo* at room temperature. Both methods showed close agreement.

Nitrogen. This determination was made by the Kjeldahl method. The values reported are corrected to a 'dry' basis, but, as no ash determinations were made, they are not corrected for mineral impurities.

Nitrogen distribution. This determination was restricted to the estimation of groups which could be used to determine whether a given preparation was gliadin, glutenin, or a mixture of these two proteins. Gliadin and glutenin, from a chemical standpoint, differ chiefly in their content of amide, arginine, and proline nitrogen. The first two of these can be determined with fair accuracy by modifications of the Van Slyke (47, 48) method. The proline nitrogen, however, cannot be estimated in this way with any degree of accuracy. Attempts to determine this form of nitrogen more directly, using a modification of Dakin's (11, 12) butyl alcohol extraction method, were also abandoned as inaccurate. Consequently, the basic, total filtrate, and filtrate amino nitrogen were determined in the usual manner by precipitation with phosphotungstic acid, although this method is subject to considerable error. A brief description of the procedure will now be given.

The proteins were hydrolyzed for 24 hr. with 6 cc. of 20% hydrochloric acid per gram of protein. The hydrolysate was then evaporated to remove most of the acid, diluted, and the acid-insoluble humin filtered off. The hydrolysate was then made up to such a volume that it would contain approximately 1 mg. of nitrogen per cc. A small amount of toluene was added as a preservative. As subsequent analyses were, in most cases, carried out on 100-cc. portions of this hydrolysate, adequate replication of all the analyses could be made from one hydrolysis. The amide nitrogen was determined by the aëration method recommended by Plimmer and Rosedale (38), except that sodium

carbonate was used instead of lime. Arginine determinations were made directly on this residue by the method of Plimmer and Rosedale (39). The precipitations with phosphotungstic acid were carried out on other residues from the amide determination in essentially the same manner as that recommended by these authors (38). The precipitate of basic nitrogen was transferred directly to a Kjeldahl flask, since no attempt was made to estimate the individual bases. Arginine determinations were carried out on an aliquot portion of the filtrate and the basic and filtrate fractions corrected for the unprecipitated arginine. No correction was applied for the solubility of the other bases.

Source of Proteins

The flour, from which the following protein preparations were made, was milled in the laboratory from a high-grade sample of Marquis, a variety of hard red spring wheat. This flour was free from bleaching agents and chemical improvers.

Preliminary Experiments

The hydrogen ion concentration of a number of urea solutions was determined at the outset, since they tend to decompose and become alkaline even at room temperature. A quinhydrone electrode gave 6.6 and 7.3 as the extreme pH values for the samples tested. In making the protein preparations, urea solutions more alkaline than pH 6.8 were adjusted to this value by the addition of dilute acetic acid. It was found that the hydrogen ion concentration of a 30% urea solution did not change significantly on standing two days. This gave ample time for centrifuging dispersions and making the other manipulations necessary at room temperature. Either Baker's or Merck's C.P. urea was used in the various experiments.

It was found that gluten could be dispersed in urea solutions of over 20% concentration. However, the lower concentrations dispersed the gluten so slowly that a 30% solution was adopted. The moisture content of a number of well-washed glutens from this flour was found to be about 60%. This was assumed to be the moisture content of subsequent glutens, and enough solid urea was added to each dispersion to bring the water contained in the gluten up to a concentration of 30% urea.

A concentrated dispersion of gluten in urea solution has a yellow, milky appearance, which remains opalescent on diluting the dispersion with more 30% urea. In the preparation of glutenin by the alkali method, Osborne (36) strongly emphasizes the necessity of filtering the dispersion clear to remove the impurities. This was attempted with the urea dispersion, but was found impossible. Either the dispersions would not filter, or, if they did, they yielded only a small amount of clear liquid which contained very little protein.

Gluten which had been extracted with alcohol to remove the gliadin was found to be entirely insoluble in 30% urea solution. To avoid this prolonged exposure to alcohol the gluten was dispersed in urea solution and alcohol added to precipitate the glutenin. Precipitation took place at an alcohol concentration of about 70% (by volume) of ethyl, and at about 60% of methyl alcohol, but only part of the resulting precipitates could be redispersed in urea. Since

Hardy and Gardner (21) report that the serum proteins can be purified with alcohol without being coagulated at temperatures of about 0° C., experiments were performed in which the precipitate was kept below 5° C. until the alcohol was removed. The precipitate obtained in this way was more soluble in 30% urea, but the amount of insoluble material was still too large to make this a feasible method for isolating glutenin.

The next method employed for precipitating the glutenin from a urea dispersion of gluten was that of 'salting-out', the method commonly used for separating serum albumin and globulin. Csonka and Jones (10) have applied this method of precipitation to alkali dispersions of gluten. They report that the gliadin and most of the glutenin is precipitated from a 0.2% solution of sodium hydroxide at a salt concentration of 0.018 of saturation with ammonium sulphate. The rest of the glutenin, which is comparatively small in amount, is precipitated at a salt concentration of 0.18 of saturation. It was thought that a salt of lower precipitating power than ammonium sulphate might facilitate the separation of the gliadin and glutenin. Magnesium sulphate was therefore selected for trial.

To determine the concentration of magnesium sulphate required to precipitate the protein from 30% urea solution, an approximately 5% dispersion of gluten was divided into 100-cc. portions and adjusted to various salt concentrations by the addition of 30% urea saturated with magnesium sulphate. The salt concentration at which the first precipitation took place is given in Table I. This precipitation coagulated all the yellow, opaque material, and left an almost clear supernatant liquid which gave a copious precipitate on the addition of tannic acid. This experiment was carried out at room temperature and the pH of the solution was 6.9 before adding the salt solution and 6.8 after its addition.

The values reported in Table I cannot be regarded as applying under all conditions. Doubtless, the salt concentration required to cause precipitation varies with the urea concentration, the hydrogen ion concentration, and the temperature at which the precipitation is carried out. The effect of these factors was not studied. Later, in making preparations by this method, it was found difficult to obtain any coagulation below a salt concentration of

TABLE I

CONCENTRATION OF MAGNESIUM SULPHATE REQUIRED TO PRECIPITATE GLUTENIN FROM 30% UREA DISPERSIONS OF GLUTEN*

Sample No.	Salt concentration expressed in terms of saturation	Time required for precipitation	Sample No.	Salt concentration expressed in terms of saturation	Time required for precipitation
1	0.13	Overnight (partial)	5	0.16	2 hr.
2	0.14	Overnight	6	0.17	15 min.
3	0.15	2 hr.	7	0.18	15 min.
4	0.15	2 hr.	8	0.19	Immediately

*These samples were made up from ordinary washed gluten containing some starch.

0.15 of saturation. The presence of starch may also influence the precipitation limit, as gluten dispersions free from starch were, in later work, found to require a salt concentration of 0.18 to bring about a satisfactory precipitation in one hour.

The protein precipitated by magnesium sulphate, in the concentrations reported in Table I, was almost entirely insoluble in 60% alcohol. This was taken as initial proof of its being glutenin. Furthermore, the supernatant liquid gave no precipitate when adjusted to an alcohol concentration of 70% by volume, this indicating that the protein which remained dispersed was largely gliadin.

The efficacy of this method of separating the two proteins depends on the salt concentration at which gliadin starts to precipitate. To test this point, a sample of gliadin was dissolved in 30% urea in 1, 3, 7 and 10% concentrations. All of these dispersions were perfectly clear. A solution of 30% urea saturated with magnesium sulphate was then added to these dispersions, and the salt concentrations which caused (a) permanent turbidity, and (b) definite slight precipitation, were noted. The results obtained are reported in Table II. It is evident that the point of permanent turbidity can be more accurately determined and is the more important, because gliadin in that condition would be swept down by the flocculating glutenin.

TABLE II

CONCENTRATIONS OF MAGNESIUM SULPHATE REQUIRED TO PRECIPITATE GLIADIN FROM 30% UREA SOLUTIONS

Concentration of prepared gliadin, %	Salt concentration expressed in terms of saturation	
	Permanent turbidity	Definite slight precipitation
1	0.15	0.18
3	0.14	0.18
7	0.14	0.16
10	0.13	0.14

The results given in Table II show that the salt concentration required to cause permanent turbidity depends somewhat on the concentration of the gliadin. However, all concentrations become turbid at a salt concentration below that required to precipitate glutenin. This result suggests that the lowest salt concentration which will cause precipitation of the glutenin will be most effective in removing the gliadin. In subsequent work, each dispersion of gluten was titrated to the salt concentration that would just give precipitation of the glutenin in from one to two hours.

The glutenin precipitated by salting-out was readily dispersed by 30% urea solution after washing out the salt with distilled water. A small amount of denatured material would result from each salt precipitation but it was much less than that obtained by precipitation with alcohol. Glutenin which had been purified by three precipitations with salt was allowed to stand in 60% alcohol for extended periods, with occasional shaking. The first extracts so

obtained gave a faint test for gliadin. As subsequent extractions with 60% alcohol gave negative tests for soluble nitrogen it was concluded that salting-out was an effective method of separating the two proteins.

It was found that the urea could not be completely removed from the last protein precipitate by extraction with 60% alcohol at room temperature. If the precipitate was exhaustively washed with water, however, the nitrogen content of the protein indicated the absence of urea. The possibility of urea remaining sorbed on the protein was tested further by extracting the washed protein with water at 60° C. for several hours. Tests for urea made on the aqueous extracts thus obtained were negative, indicating that the adsorbed urea which survived the washing would not affect appreciably the chemical composition of the protein.

Even when the gluten was extremely well washed, the dispersions showed the presence of considerable amounts of starch. This would settle out partly on standing several days, but even then the dispersion gave a strong test for starch. Ordinary centrifuging was of little value in reducing this starch test, so the starch was regarded as being dispersed, in accordance with the work of Foulger (15), and its removal at this stage was abandoned in favor of its removal later by precipitation. In isolating the glutenin by salting-out, the strong starch test of the original gluten dispersion was reduced to a faint test in the dispersion obtained from the first precipitate, while the dispersion from the second precipitate usually gave a negative starch test. This suggested that the starch was less soluble than the glutenin, and remained behind in the small amount of insoluble material. Nevertheless the nitrogen content of these preparations was usually lower than the 17.49% reported by Osborne (37) for glutenin. However, as the preparations appeared to be free from starch, no further improvements in the method were attempted at this stage.

Several efforts were made to obtain dry glutenin which would redisperse in 30% urea. All the samples that were dehydrated with alcohol and ether were denatured, as has been noted previously. Drying at low temperature without the use of alcohol resulted in a similar insolubility. Two small preparations were then dried carefully at room temperature, using an air blast and grinding the material through a food chopper several times to facilitate drying. When tested in the air-dry condition, they were found to be entirely insoluble in 30% urea solution. As these preparations were originally quite soluble, the only apparent denaturing influence was that of drying the material. This suggests that the change involved in the denaturation of glutenin may be some form of dehydration.

Description of Preparations

Having given the method of preparation some study, the isolation of a sample of glutenin large enough for subsequent analysis was undertaken.

Preparation 1. About 300 gm. of well-washed wet gluten was torn up and placed in 1700 cc. of 30% urea solution, and sufficient solid urea added to bring the water in the gluten up to a urea concentration of approximately 30%. This was allowed to stand for 24 hr. at room temperature with occasional

stirring. The small amount of undispersed material was removed by centrifuging and the dispersion adjusted to a salt concentration of 0.14 of saturation by adding 30% urea saturated with magnesium sulphate. At this salt concentration, no immediate coagulation was noticeable, but it precipitated on standing overnight, leaving a slightly turbid supernatant liquid which was decanted. The residue was then washed repeatedly with water, replaced in 1500 cc. of urea solution (again adding sufficient solid urea to adjust the water in the precipitate to approximately 30% urea), allowed to stand 24 hr. to disperse, and then centrifuged to remove the insoluble material. As the starch test in this dispersion was almost as marked as in the original dispersion, sufficient salt solution was added to make the dispersion 0.16 saturated, to see if this would render the carbohydrate impurities less soluble. Precipitation occurred immediately, leaving a clear supernatant liquid. The precipitate was washed with water several times, redispersed in 30% urea, and finally centrifuged to remove the insoluble material. The starch test was much weaker this time, and after another precipitation at 0.16 of saturation, this test was negative.

The supernatant liquid from this precipitation was adjusted to a salt concentration of 0.20 of saturation. A small quantity of protein was precipitated, which was washed and dehydrated. This sample gave a negative starch test, a positive Molisch test, and contained 16.7% nitrogen.

The precipitate obtained at 0.16 of saturation was redissolved in 30% urea and precipitated by dilution, using five volumes of distilled water. This method of precipitation was used to facilitate the removal of the magnesium sulphate and urea. The precipitate came down in a white flocculent form, which was easily broken up by shaking. It was washed with water until free from the sulphate ion and urea. The sample thus obtained was extracted for two days with 60% alcohol, the water in the precipitate having been adjusted to this concentration with 95% alcohol. This extract gave a faint test for gliadin, but a second 60% alcohol extract was protein-free. The sample was then dehydrated with absolute alcohol and anhydrous ether, and finally dried over phosphorus pentoxide *in vacuo*. The sample obtained was a white, finely divided powder. It weighed nine grams, which corresponds to about 10% of the weight of dry gluten used. This preparation gave a negative starch test, a positive Molisch test, and contained 16.4% nitrogen. A further analysis of this sample is given in Table IV.

Judging from the appearance of the protein, the single precipitation by dilution in the last preparation effected more purification than a single precipitation with salt. If this method were used throughout, however, the removal of gliadin would depend on its solubility in dilute solutions of urea. Dill and Alsberg (13) tested the solubility of 1% of gliadin in urea solution and obtained a turbidity on dilution to 2.03 molar (12%). Observations made during the course of this investigation suggested that gliadin was soluble in urea solutions more dilute than this. A test, conducted to determine the solubility of gliadin in 10% urea, showed that this urea concentration would disperse 5% of gliadin to an extremely viscous, clear sol. On dilution, this dispersion

became turbid, but the resulting precipitate represented only part of the original protein.

These results with prepared gliadin made it appear that not only was precipitation by dilution a feasible method of separating the two proteins, but that an initial extraction with 10% urea would be helpful in removing the gliadin. A test preparation made by extracting gluten six times with 10% urea solution, followed by two dispersions and dilution precipitations from 30% urea, yielded a sample which gave a negative test for starch and contained 16.4% of nitrogen. The amide and arginine nitrogen contents of this preparation were respectively 20 and 10% of the total nitrogen.

Preparations 2 and 3. The preparation of a larger sample by this method was then undertaken. Unintentionally, the protein was fractionated when the first precipitation was made, resulting in two preparations of glutenin.

These preparations were started by extracting 600 gm. of wet gluten with 10% urea solution. Six extractions were made, using about 1500 cc. of urea solution for each extraction, which was of 12 hr. duration, the material being stirred frequently. After the sixth extraction, 2500 cc. of 30% urea solution was added, and the remaining material allowed to disperse for 24 hr. at room temperature. A small amount of insoluble material was then thrown down in an ordinary centrifuge, and the dispersion diluted with two volumes of distilled water. As no precipitation took place, another volume of distilled water was added, thus diluting the urea concentration to about 7.5%. As this did not cause precipitation, the equivalent of 0.5 gm. per litre of sodium chloride was added. This gave a white flocculent precipitate which was removed by decantation, and is subsequently designated as Preparation 2.

The supernatant liquid from the above precipitation was quite opaque and appeared to contain considerable protein. A further addition of 0.5 gm. per litre of salt resulted in a precipitate similar to the first. This also was obtained by decanting off the liquid, and was kept separate from the first precipitate and designated Preparation 3.

After the removal of the second precipitate, the supernatant liquid was not entirely clear, so a third addition of salt was made. A small amount of precipitate resulted, which was purified and dehydrated, but was not allotted a preparation number as it was later found to be largely gliadin. The first indication of this was that much of it was lost in subsequent purification owing to its solubility in 10% urea. The product obtained from this third precipitation, when dehydrated, was found to contain only 15.7% nitrogen, which indicated the presence of impurities, although the starch test was negative. A small amount was hydrolyzed and yielded 27.1% of its nitrogen as ammonia, which definitely established that it was gliadin.

The subsequent purification of Preparations 2 and 3 will now be described together, since it was essentially the same for both samples. The precipitates from the fractional precipitations were dissolved in urea solution, centrifuged, and again diluted with two volumes of distilled water. As no precipitate came down, and since further fractionation was undesirable, 2 gm. per litre of salt was added. This gave immediate precipitation, leaving a

clear supernatant liquid. Both precipitates were then washed with 10% urea solution. As the first two of these washings dispersed sufficient protein to make them quite opaque, they were precipitated and purified separately. The results of such analyses as could be performed on these samples are shown in Table III, together with similar analyses of the preparation from which they were obtained.

After four washings with 10% urea solution, the material from the second precipitation was redissolved in 30% urea, centrifuged, and reprecipitated as before, except that lithium chloride instead of sodium chloride was emplo ed. The precipitates were then washed once with 10% urea, which appeared to remove but little protein, and then with water until the washings gave no test for the chloride ion or urea. The samples were then dehydrated with alcohol and ether. The 60% alcohol extractions gave a faint test for gliadin on the first washing only. Other tests conducted on the dry samples are reported in Table III, and a more comprehensive analysis given in Table IV.

TABLE III

STARCH AND MOLISCH TESTS AND NITROGEN CONTENT OF PREPARATIONS 2 AND 3 AND EXTRACTS OBTAINED FROM THEM

Nature of sample	Preparation 2			Preparation 3		
	Starch test	Molisch test	Nitrogen content, %	Starch test	Molisch test	Nitrogen content, %
Protein in 1st 10% urea extract	−	?	17.0	−	?	17.0
Protein in 2nd 10% urea extract	−	?	16.7	−	?	16.7
Purified preparation	−	+	15.8	−	?	16.3

It is evident from Table III that the nitrogen contents of the proteins extracted by 10% urea solution are higher than those of the purified preparation. This suggests that some impurity of lower solubility than the glutenin was present in the material. Tests for starch were negative, but Preparation 2 gave a positive Molisch reaction. The rest of these fractions, when tested for carbohydrate material, gave such a faint test that the presence of impurities of this nature could not be definitely established. It is probable that the carbohydrate impurity in these preparations was the gum mentioned by Hoffman and Gortner (23).

The behavior of starch in 30% urea was given some consideration at this point. It has been mentioned that a gluten dispersion in urea gives a strong test for starch, which was regarded as being dispersed, since no appreciable amount could be settled out by prolonged centrifuging in an ordinary centrifuge. To test this assumption, a sample of starch prepared from the same flour was placed in 30% urea solution. This starch swelled considerably, but even on standing ten days with occasional shaking, a water-clear supernatant liquid remained. This clear liquid gave a test for starch but it

appeared to be small in amount compared with the undispersed material. The starch, and other impurities affecting the nitrogen contents of Preparations 2 and 3, appear, therefore, to be less soluble than the protein. This result, together with the data presented in Table III, indicated that the carbohydrate impurities were less easily dispersed than the protein. It should, therefore, be possible to remove them. Ordinary centrifuging, however, was of little value; filtering the dispersion was so slow as to be impracticable, and also appeared to remove most of the glutenin.

At this stage, a Sharples supercentrifuge was made available for this work. A gluten dispersion passed through this machine at a rate of 50 cc. per minute gave a negative starch test, and on subsequent purification the glutenin so obtained contained 17.1% of nitrogen. As this procedure was apparently effective in removing the impurities, the preparation of further samples was undertaken.

Preparation 4. A dispersion of gluten was prepared by placing 400 gm. of wet gluten, plus the calculated amount of solid urea, in four litres of 30% urea solution. This was stirred for two hours at room temperature, and then allowed to stand for 18 hr. at 0° C. in a refrigerator. The dispersion was then supercentrifuged, as described above. About 4200 cc. of a starch-free protein solution was thus obtained. This was divided into two equal parts, one of which was used for making Preparation 5, to be described later.

Preparation 4 was made by salting-out the glutenin. The dispersion described above was adjusted, at intervals of about one hour, to salt concentrations of 0.14, 0.15, 0.16, 0.17, and finally to 0.18 of saturation, at which salt concentration flocculation took place. This precipitate was allowed to settle overnight at 0° C. It was then washed three times with distilled water and redispersed in 1500 cc. of 30% urea. The protein dispersed readily, but it was allowed to stand 12 hr. before supercentrifuging it again. This removed a small amount of material which appeared to be protein. Precipitation of this dispersion took place at a salt concentration of 0.16 of saturation. The precipitate was allowed to settle, washed, redispersed, supercentrifuged, and again precipitated at a salt concentration of 0.16 of saturation. It was then washed with water until free from urea and the sulphate ion. This was followed by three prolonged extractions with 60% alcohol, the first two of which gave a faint test for gliadin. The preparation was then dehydrated. About six grams of protein was obtained, which is a lower yield than that obtained in Preparation 1. This agrees with the observation that more of the precipitated material became insoluble during the purification process. This may be attributed to the higher salt concentration used for the first precipitation, or to the more effective removal of the denatured material by the supercentrifuge. The nitrogen content of this preparation was 17.0%; further analyses are given in Table IV.

Preparation 5. This preparation was obtained by precipitating the glutenin by dilution. Half of the starch-free dispersion described under Preparation 4 was used. To avoid fractionation in the first precipitation, this dispersion was poured into three volumes of 1% lithium chloride solution. Precipitation

took place immediately as a coherent, gluten-like mass, which could not be washed satisfactorily; so after two attempts, it was redispersed in 30% urea solution. After allowing 24 hr. for complete dispersion, it was supercentrifuged and precipitated by pouring it into three volumes of distilled water. This also resulted in a gluten-like precipitate, which could not be washed readily. Apparently, this coherent precipitate was partly due to the large amount of gliadin present, which had been to a large extent removed from Preparations 2 and 3 by the initial extraction with 10% urea. This precipitate was then washed twice with 10% urea before replacing it in the more concentrated solution. On standing overnight, dispersion was complete and it was again supercentrifuged and precipitated by the careful addition of water to the dispersion. This gave a fine, white precipitate, which flocculated readily. The urea concentration at which this precipitation occurred was approximately 12%. The precipitate was washed with 10% urea and subjected to one more dispersion and precipitation before washing it free from urea and dehydrating it in the usual manner. The first two extractions with 60% alcohol gave a faint test for gliadin. Somewhat more denaturation was observed in this preparation than in Preparations 2 and 3. About five grams of dry protein was obtained, which contained 17.3% nitrogen. Further analyses are reported in Table IV.

Preparation 6. A small amount of glutenin was isolated by solution in alkali for comparing the nitrogen distribution of such a preparation with those isolated by dispersion in urea solutions. After exhaustive extraction of the gliadin with 60% alcohol, the remainder of the gluten was dispersed in 0.2% sodium hydroxide. This dispersion was supercentrifuged and the protein precipitated by neutralization with 0.5 *N* hydrochloric acid. Three such precipitations were made in all, each precipitate being washed with 60% alcohol before redissolving in alkali. This preparation also gave a faint test for gliadin in the first two 60% alcohol washings from the final precipitate. This preparation contained 16.4% nitrogen.

Analytical Results

The values for the nitrogen content and nitrogen distribution of the glutenin preparations just described are given in Table IV. Preparations 4 and 5, which were supercentrifuged to remove the starch and other impurities, have distinctly higher nitrogen contents than the first three preparations. The reported values are on a moisture-free basis, but corrections were not made for their mineral content. Ash determinations on Preparations 4 and 5 showed less than 0.4% ash, an amount which would not affect the nitrogen percentages appreciably. Osborne (37) gives the nitrogen content of glutenin as 17.49%, which is significantly higher than any of these preparations. It must be concluded either that these preparations contained non-nitrogenous impurities, or that Osborne's value is too high. The latter is at least possible, since the prolonged alcoholic extraction of the older methods of preparation might have denatured some of the less soluble fractions of the gliadin.

TABLE IV

NITROGEN CONTENT AND DISTRIBUTION OF GLUTENIN PREPARATIONS

Preparation No.	Dispersion medium and method of preparation	Nitrogen content	Nitrogen distribution								
			Humin N	Amide N	Arginine N	Phosphotungstic acid precipitation					
						Arginine N in filtrate	Values for fractions corrected for arginine nitrogen in filtrate				Recovery
							Basic N	Total filtrate N	Filtrate amino N	Filtrate non-amino N	
		%	%	%	%	%	%	%	%	%	%
1	Urea dispersion salted out	16.4	0.6	16.4	11.1	2.7	21.4	62.1	54.7	7.4	99.9
2	Urea dispersion diluted	15.8	1.3	19.4	9.9	2.5	18.6	61.0	57.4	3.6	99.0
3	Urea dispersion diluted	16.3	0.7	19.9	9.6	2.7	17.9	60.8	54.8	6.0	99.3
4	Urea dispersion salted out*	17.0	0.3	19.3	9.8	5.0	17.5	61.2	60.7	0.5	98.3
5	Urea dispersion diluted*	17.3	0.4	19.5	10.0	3.8	17.8	61.6	60.3	1.3	99.3
Average of preparations 2, 3, 4 and 5				19.5	9.8		18.0	61.2			
6	NaOH neutralized	16.4	0.5	17.9	11.0	5.0	23.2	57.7	57.7	0.7	99.3

**These preparations were supercentrifuged to free them from carbohydrate and other impurities.*

All values given in Table IV for the nitrogen distribution are expressed as a percentage of the total nitrogen in the hydrolysate. It is evident that the last four urea preparations (Preparations 2 to 5) are essentially the same in their contents of amide and arginine nitrogen. They are also similar in their basic and total filtrate nitrogen contents, although less accuracy is claimed for these figures. The variations between these preparations in filtrate amino nitrogen may signify real differences, but, considering the errors inherent in such a determination, and the similarity of the preparations with respect to the other nitrogen fractions, the variations are probably not significant. Since the non-amino nitrogen is subject to more error than any of the other fractions, its variability in the different preparations cannot be taken as establishing the fact that there are any real differences between them.

Preparation 1 differs significantly from the other urea preparations in having a lower amide and a higher arginine nitrogen content, resembling in this respect the alkali preparation (Preparation 6). There is no evident reason for this preparation differing from the others, but it is probable that it was denatured or otherwise altered to some extent, since it was the first sample isolated by the urea method. Blish and Sandstedt (5) have found that the amide nitrogen content of glutenin decreases, and the arginine nitrogen increases with the concentration of alkali used for its dispersion. If it can be inferred that the higher concentrations of alkali alter the glutenin more than lower concentrations, then a decrease in amide nitrogen and an increase in arginine nitrogen represent a greater degree of alteration. From this reasoning, Preparations 1 and 6 appear to be altered in comparison with the other urea

preparations. While this is probably so, no reason can be given for this alteration in the case of the urea preparation. The similarity between the arginine and amide nitrogen contents of the last four urea preparations, which includes precipitation by salting-out and by dilution, leads to the conclusion that glutenin is at least a chemical individual.

In Table V some of the values reported in the literature for the amide and arginine nitrogen of glutenin are presented for comparison with the preparations just described. The values for the other fractions obtained in the determination of the nitrogen distribution are not compared, as they are subject to greater error, especially in comparing the work of different investigators. The values obtained by any one investigator have been averaged, regardless of the source of the glutenin. This is permissible, since the individual workers have interpreted their results as showing no significant variation between the glutenins from different flours. Further, Blish and Sandstedt (5) have shown that the percentage of amide and arginine nitrogen is affected by the concentration of alkali used in dispersing the glutenin. The values for the four urea preparations having essentially the same composition in regard to the constituents concerned have also been averaged. The data obtained from Preparations 1 and 6 are presented separately.

It is evident that the composition of Preparations 1 and 6 come well within the range of values obtained by other investigators for glutenin prepared by

TABLE V

AMIDE AND ARGININE NITROGEN CONTENTS OF GLUTENIN

Observer	Method of isolation	No. of preparations analyzed	Amide N %	Arginine N %
Osborne 1909 (37)	Alkali dispersion	—	18.8	—
Blish 1916 (3)	Alkali dispersion	2	16.4	9.5
Cross and Swain 1924 (9)	Alkali dispersion	4	14.9	10.0
Hoffman and Gortner 1927 (23)	Alkali dispersion	1	13.6	12.0
Larmour 1927 (30)	Alkali dispersion	1	14.8	10.9
Csonka and Jones (α-glutelin)* 1927 (10)	Alkali dispersion	1	17.8	11.0
Blish and Sandstedt 1929 (5)	Acid dispersion	9 or 10	about 22	about 9
Authors	Alkali dispersion (Prep. 6)	1	17.9	11.0
Authors	Urea dispersion (Prep. 1)	1	16.4	11.1
Authors	Urea dispersion (Preps. 2 to 5)	4	19.5	9.8

*The values for the β-glutelin are not given as it apparently occurs in small quantity.

the alkali method. As Preparation 6 was prepared from alkali, and Preparation 1, though isolated by the urea method, was apparently altered in some way, these preparations will not be discussed further. The amide nitrogen content of the other glutenin samples prepared by the urea method is significantly higher than all the preparations made by the alkali method. Since no urea could be detected in an aqueous extract of the protein made at 60° C., it appears that the high amide nitrogen of the urea preparations cannot be attributed to urea remaining sorbed on the protein. The glutenin preparations of Blish and Sandstedt (5), made by dispersion in acid, are the only ones having a higher amide nitrogen content than the urea preparations. Considering the arginine nitrogen of glutenin, it is evident that the urea preparations give values which are intermediate between those of the acid dispersion and those of the alkali dispersion preparations, except in the case of the alkali preparations reported by Blish (3).

It appears from these results that the hydrogen ion concentration of the dispersion medium used for preparing the glutenin has some effect on its subsequent amide and arginine nitrogen values. This agrees with the work of Knaggs and Schryver (27), and Knaggs (26) who found that acid- and alkali-treated collagens differed in their content of diamino nitrogen. This is of special interest, since collagen resembles glutenin in being insoluble in the neutral solvents commonly used for dispersing proteins. The results of chemical analyses performed on such proteins as glutenin should therefore be more reliable when a neutral dispersion medium is employed for their purification.

Possible Denaturation by Urea Solutions

Since concentrated urea solutions are known to denature certain proteins, attempts were made to determine their effect on glutenin. This is a difficult point to test experimentally by the usual solubility methods since glutenin is originally insoluble in the ordinary neutral solvents. In making the preparations just described, a small amount of protein remained insoluble after each precipitation, but this takes place in the preparation of most proteins. Physical tests for determining the denaturing effect of urea solutions on gluten were conducted by dispersing washed gluten in 30% urea solution, supercentrifuging to remove the starch, and then determining the viscosity of the dispersion after storage for various periods at 5° C. The hydrogen ion concentration was adjusted to pH 6.95 with phosphate buffer mixtures. No change in viscosity was observed for periods up to 100 hr., the longest storage time employed. There was no evidence of precipitation even when the dispersions were stored for much longer periods. It should be noted, however, that the gluten was completely dispersed before any viscosity measurements were made. According to Hopkins (24) urea denaturation takes place rapidly, and as it has a negative temperature coefficient, it is possible that the gluten was altered before the initial viscosity was observed. Further studies on viscosity changes in relation to the denaturation of the gluten proteins will be presented in a later paper.

Harris (22) and more recently, Hopkins (24) have shown that such proteins as egg albumin, when denatured either by heat or by concentrated urea solutions, give a positive test for a thiol group when treated with sodium nitroprusside. Ramsden's (42) results indicate that it is necessary to remove the urea from the protein before making the nitroprusside test for denaturation.

The first tests were made on flour and wet gluten in order to establish the validity of this test as a criterion of denaturation in the gluten proteins. The results obtained with flour were inconclusive, due to their large starch content, but if any color developed it was extremely faint. Wet gluten gave a doubtful test at first but a deeper color gradually developed on standing, a result which may be attributed to denaturation caused by the weak alkaline solution in which the test is made. This test was then applied to the preparations described in Table IV which had, of course, been denatured by drying. The urea preparations all gave a faint test, but the alkali preparation gave a more intense color than any of these. Some samples of gliadin prepared in the usual manner, which does not involve exposure to alkali or urea, gave tests of similar intensity. Further tests were made on gluten dispersed in (a) dilute sodium hydroxide, (b) dilute acetic acid, and (c) 30% urea for 48 hr. and then dialyzed. The protein from the alkali dispersion gave a much deeper color than the protein dispersed in acetic acid or urea, the two latter giving about the same shade.

Although these tests must be regarded as of a preliminary nature, they furnish evidence to show that the thiol test is indicative of denaturation in the gluten proteins. All of the samples of prepared gliadin and glutenin, subjected to test, gave a faint positive reaction, indicating that the purification process had caused some denaturation. Judging from these results, glutenin prepared by the urea method is altered less than samples prepared by solution in alkali.

Discussion and Conclusions

At the present time, it cannot be stated definitely whether salting-out or dilution is the better method of precipitating glutenin from urea dispersions. A small amount of denatured material occurs with both methods. It is probable, however, that precipitation by dilution yields a product with a lower content of mineral impurities. If this method of precipitation is employed, it appears to be advantageous to extract thoroughly with 10% urea solution first, in order to remove most of the gliadin. This renders the subsequent precipitates less coherent and consequently facilitates the washing of the material.

Whatever the method of precipitation used, it is necessary to remove the carbohydrate impurities at an early stage. This can be accomplished by passing the 30% urea dispersions slowly through a Sharples supercentrifuge. Usually three precipitations suffice to remove the gliadin from the glutenin, provided each precipitation is made under conditions which will just cause the glutenin to coagulate. Any excess of the precipitating agent will cause more of the gliadin to be thrown down with the glutenin; the latter protein in consequence will have to be precipitated more often to insure purity. When the removal of the gliadin is completed, the precipitate is washed by shaking vigor-

ously with water at 0° C. until the washings contain no measurable amount of nitrogen, and then washed several times with water at room temperature, the urea appears to be completely removed from the protein.

Glutenin loses its original solubility in 30% urea solution if it is exposed to alcohol or dried; such treatments must therefore be avoided in making preparations for physical study. Hitherto, dilute alkalies and acids have been used for the preparation of glutenin. Judging from the sulphydryl test, urea solutions cause less denaturation than dilute alkalies, and are not inferior to acid in this respect. In contrast to the alkali preparations, different samples of glutenin isolated by the urea method have practically similar amide and arginine nitrogen contents. The amount of these constituents is intermediate between those reported for preparations made by acid and alkali. Urea solutions, being neutral, also permit a study of the physical properties of glutenin in the region of its normal isoelectric condition without previous exposure to extremes of hydrogen ion concentration.

Proteins of the glutelin class occur in many of the cereal grains, such as wheat, oats, barley, corn, etc. Of these grains, only wheat yields a gluten from which most of the starch can be removed by kneading in a stream of water. In preparing the glutelins from the other cereals, it is, therefore, necessary to extract the protein directly from the flour or ground material. Since solutions of urea apparently disperse the protein more readily than the starch, it would seem that the urea method just described could be adapted to the preparation of glutelins from other cereals. The application of this method to the preparation of other glutelins would doubtless lead to a better understanding of these proteins; at least, it would avoid the alterations caused by prolonged contact with alkali and alcohol.

Acknowledgment

The authors wish to thank Dr. R. Newton, Professor of Field Crops, University of Alberta, for helpful suggestions made during the course of this investigation and for his criticism of the manuscript.

References

1. Alsberg, C. L. and Perry, E. E. "The preparation of glutenin". Abstract circulated for Los Angeles meeting of the American Chemical Society, Aug. 1925.
2. Anson, M. L. and Mirsky, A. E. J. Gen. Physiol. 13: 121-132. 1929.
3. Blish, M. J. J. Ind. Eng. Chem. 8: 138-144. 1916.
4. Blish, M. J. Cereal Chem. 2: 127-131. 1925.
5. Blish, M. J. and Sandstedt, R. M. J. Biol. Chem. 85: 195-206. 1929.
6. Burk, N. F. and Greenberg, D. M. J. Biol. Chem. 87: 197-238. 1930.
7. Chick, H. and Martin, C. J. J. Physiol. 43: 1-27. 1911.
8. Chick, H. and Martin, C. J. J. Physiol. 45: 261-295. 1913.
9. Cross, R. J. and Swain, R. E. Ind. Eng. Chem. 16: 49-52. 1924.
10. Csonka, F. A. and Jones, D. B. J. Biol. Chem. 73: 321-329. 1927.
11. Dakin, H. D. Biochem. J. 12: 290-317. 1918.
12. Dakin, H. D. J. Biol. Chem. 44: 499-529. 1920.
13. Dill, D. B. and Alsberg, C. L. J. Biol. Chem. 65: 279-304. 1925.
14. Fischer, M. H. Oedema and Nephritis, Wiley, N.Y. 1915.

15. FOULGER, J. H. Med. Bull. Univ. Cincinnati, 3: 46-47. 1924.
16. FÜRTH, R. Ann. Physik, 70: 63-80. 1923.
17. GARREAU, Y. and MARINESCO, M. N. Compt. rend. 189: 331-333. 1929.
18. GORTNER, R. A., HOFFMAN, W. F. and SINCLAIR, W. B. Colloid Symposium Monograph, 5: 179-198. 1928.
19. HALTON, P. J. Agr. Sci. 14: 587-599. 1924.
20. HARDY, W. B. J. Physiol. 24: 158-210. 1899.
21. HARDY, W. B. and GARDNER, S. J. Physiol. 40: lxviii. 1910.
22. HARRIS, L. J. Proc. Roy. Soc. Lond. 94B: 426-441. 1923.
23. HOFFMAN, W. F. and GORTNER, R. A. Cereal Chem. 4: 221-229. 1927.
24. HOPKINS, F. G. Nature, 126: 328-330, 383-384. 1930.
25. HUANG, T. C. and WU, H. Chinese J. Physiol. 4: 221-230. 1930. Quoted by Chem. Abs. 24, 4061. 1930.
26. KNAGGS, J. Biochem. J. 23: 1308-1327. 1929.
27. KNAGGS, J. and SCHRYVER, S. B. Biochem. J. 18: 1095-1101. 1924.
28. KNAGGS, J. and SCHRYVER, S. B. Biochem. J. 18: 1102-1106. 1924.
29. KOCKEL, L. Ann. Physik, 77: 417-448. 1925.
30. LARMOUR, R. K. J. Agr. Research, 35: 1091-1120. 1927.
31 LEWIS, P. S. Biochem. J. 20: 965-977. 1926.
32. LEWIS, P. S. Biochem. J. 20: 978-983. 1926.
33. LEWIS, P. S. Biochem. J. 20: 984-992. 1926.
34. MARINESCO, M. Compt. rend. 188: 1163-1165. 1929.
35. MIRSKY, A. E. and ANSON, M. L. J. Gen. Physiol. 13: 133-143. 1929.
36. OSBORNE, T. B. Carnegie Inst. Wash. Publ. 84. 1907.
37. OSBORNE, T. B. The vegetable proteins. Longmans, Green. Lond. 1909.
38. PLIMMER, R. H. A. and ROSEDALE, J. L. Biochem. J. 19: 1004-1014. 1925.
39. PLIMMER, R. H. A. and ROSEDALE, J. L. Biochem. J. 19: 1020-1021. 1925.
40. RAMSDEN, W. J. Physiol. 28: xxiii-xxvi. 1902.
41. RAMSDEN, W. Chemistry and Industry, 49: 851-852. 1930.
42. RAMSDEN, W. Nature, 127: 403-404. 1931.
43. SJÖGREN, B. and SVEDBERG, T. J. Am. Chem. Soc. 52: 5187-5192. 1930.
44. SPEAKMAN, J. B. and HIRST, M. C. Nature, 127: 665-666. 1931.
45. SPIRO, K. Z. physiol. Chem. 30: 182-199. 1900.
46. SVEDBERG, T. Kolloid-Z. 51: 10-24. 1930.
47. VAN SLYKE, D. D. J. Biol. Chem. 10: 15-55. 1911.
48. VAN SLYKE, D. D. J. Biol. Chem. 22: 281-285. 1915.
49. WU, H. and YEN, D. J. Biochem. (Japan), 4: 345-384. 1924.

STUDIES IN THE VARIABILITY OF TUBERCLE BACILLI. IV. ANTIGENIC PROPERTIES OF S AND R CULTURES[1]

CHRISTINE E. RICE[2]

Abstract

Accompanying dissociation of certain cultures of tubercle bacilli, a correlated loss in the specific antigenic activity in the complement-fixation reaction has been noted. All extracts made from attenuated cultures in which the R colony has been shown to be predominant have been found to be inferior in antigenic activity to similar preparations made from cultures composed of S types. Antisera prepared against R organisms appear to lack antibodies against certain substances present in the S organisms, although they have been found to contain a higher proportion of antibodies reacting with related acid-fast organisms than do the corresponding S antisera. It has been necessary to employ a strictly quantitative technique to demonstrate these differences.

The work of Petroff and his associates (15, 16, 17) on the dissociation of tubercle bacilli, has been confirmed indirectly by several workers, and directly by Begbie (5) and by Reed and Rice (19, 20) in the first two papers of this series. In the latter paper, it was shown that dissociation of these organisms is accompanied by correlated changes in colony structure, in certain physical properties and in pathogenicity. Dissociation such as has been demonstrated in many bacterial species, appears to involve in addition to these changes, certain fundamental alterations in antigenic capacity, the variant type being lacking in the specific antigenic qualities which characterize the normal, freshly isolated organisms. In the colon-typhoid group of bacteria, the essential feature of the S to R change seems to be an alteration in the heat-stable somatic complex with the development of new and typical somatic antigens (White (27) and Arkwright (1, 2)). In the pneumococci and streptococci, the antigenic change would appear to be less complete, the variant although lacking the type-specific antigens, still retains the group antigen also found in the normal type (Griffith (11), Todd and Lancefield (24)). The antigenic behavior of other dissociated cultures has received less attention, but would appear to follow either of these two types of change. The present paper is concerned with a study of the antigenic behavior of certain typical S and R cultures of the tubercle bacillus.

A number of workers who have studied the complement-fixation reaction in tuberculosis have noted variation in the antigenic activity of preparations made from different strains of tubercle bacilli, Scheff (21) and Wadsworth, Maltaner and Maltaner (26) having found extracts of certain avirulent strains of these organisms to be less suitable as antigens than those made from virulent ones. Cooke (8) made the interesting observation that strains of tubercle bacilli

[1] *Manuscript received June 23, 1931.*

Contribution from the Department of Bacteriology, Queen's University, Kingston. This work was carried on with the co-operation and financial assistance of the Associate Committee on Tuberculosis of the National Research Council of Canada.

The data presented in this paper formed part of a thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy at the University of Toronto.

[2] *Fellow in Bacteriology, Queen's University.*

which were readily emulsified in saline, made the better antigens, an observation possibly related to the fact that S types are somewhat more readily emulsified than R. Shibata (22), however, could find no appreciable difference in the complement-fixing activity of alcoholic extracts prepared from the virulent Vallée and the avirulent B.C.G. strain in the presence of tuberculous serum.

Ordinary serological procedures have not been as successful to date in the differentiation of types of tubercle and other acid-fast species as have cultural methods and animal experimentation. The very close antigenic relationship within this group was demonstrated by Koch in 1901 by means of the agglutination reaction and two years later by Bordet and Gengou (6) by complement-fixation. Since that time cross reactions have been reported repeatedly (Babès and Busilia (4), Harris and Lanford (12), Cooke (8), Coulthard (10), Scheff (21), Aronson and Whitney (3), and others). Smith (23) was unable to differentiate between human and bovine tubercle bacilli by means of agglutinin absorption tests, while Wadsworth, Maltaner and Maltaner (26), Kirchner (13) and Aronson and Whitney (3) have failed to do so by means of the complement-fixation reaction. It seems evident from these results that if differences in antigenic activity between the virulent and avirulent types or between the S and R forms actually exist, more precise quantitative procedures of measurement must be adopted in order to detect them.

Of the various serological methods, complement-fixation has been found to give the most reliable results both in classification of the acid-fast species and laboratory diagnosis of tuberculous infection. The quantitative technique proposed by Mrs. Maltaner (14), in which the actual amount of complement reacting with the sensitized antigen is measured, appeared to be the most exact method so far reported and was therefore considered most suitable for our purpose.

Experimental Methods

All fundamental procedures and standardizations were carried out according to the methods outlined in Standard Methods of the New York State Department of Health, Wadsworth (25), for the complement-fixation test for tuberculosis. Preliminary tests were made in the presence of two units of complement; final tests by the method referred to above (Maltaner (14)). Twenty-four dilutions of complement ranging from a one in five or a one in ten to a one in one hundred were prepared in the same way as the complement dilutions for the daily complement titration. The final reaction in all tests was read by comparison with color standards prepared to detect differences of 5% or less in the degree of hemolysis.

(*a*) *Immune Serum*

Normal rabbits whose serum in 0.05-cc. amounts, after heating at 56° C. for one-half hour just previous to testing, did not show anticomplementary or nonspecific properties, were considered satisfactory for purposes of immunization. Before inoculation, large bleedings were taken and kept for use in parallel tests with immune serum from the same animal.

Some ten days after the animals had received the eighth intraperitoneal inoculation of vaccines prepared from killed cultures of tubercle bacilli, they were bled from the ear and the serum tested in the presence of standard S and R antigens. If good fixation was secured with 0.005 cc. or less of serum in the presence of 0.1 cc. of the optimum dilution of the standard antigens, the titre was considered satisfactory. If lower, the animal was given further inoculations.

(b) Antigens

Aqueous extracts of cultures of various strains were prepared according to the methods described in Standard Methods (25). Antigens which were still somewhat anticomplimentary in 0.02 cc. amounts after dialysis for one week, were either discarded or submitted to further dialysis.

A high titre antigen prepared from a virulent human S strain No. 13, Reed and Rice (20), which compared favorably with a carefully tested antigen made from the same strain, kindly supplied by Mrs. Maltaner, was selected as a standard S antigen. A highly active antigen prepared by a similar method from the typical R culture, No. 599, Reed and Rice (19), was chosen as a standard R antigen. The most sensitive dilutions of both of these antigens were determined by titrating in the presence of both S and R antisera, a high titre antituberculous horse serum, furnished by Mrs. Maltaner, and a No. 599 rabbit antiserum of high titre and low nonspecific activity. All new antigens were compared in parallel with the standard antigens in the presence of these two antisera.

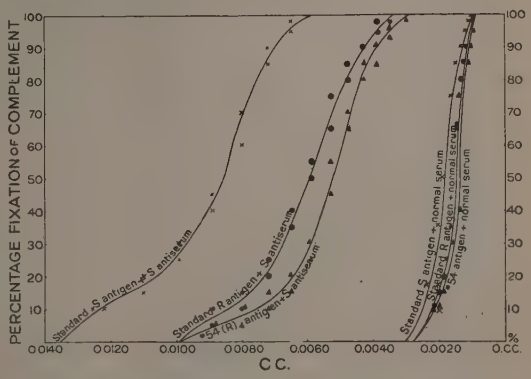


FIG. 1. *Results of a typical series of titrations of one immune and one normal serum in the presence of three different antigens (percentages inhibition of hemolysis plotted as ordinates; amount of complement fixed, as abscissas).*

(c) Final Comparative Tests

Each antigen, in 0.1 cc. amounts of the dilution found to be optimum, was tested in duplicate in the presence of various immune sera. If the serum was of high titre the serum volume was made up to 0.005 cc. by the addition of normal serum from the same animal species. Controls of the same antigen and 0.005 cc. of normal serum accompanied all tests.

The percentage inhibition of hemolysis at each dilution of complement was recorded graphically, the amounts of complement as ordinates, the percentages fixation as abscissas, Fig. I. Although the ratio between different systems was practically the same at 40, 50 and 60% hemolysis, all three points have been considered in the tables; at the extremes of 5 and 90% fixation the ratios

showed considerable variation. The specific fixation at any percentage inhibition of hemolysis was considered as the total amount of complement fixed by any one antigen in the presence of a particular antiserum less the amount of complement fixed in the presence of the same antigen and normal serum.

The standard deviation and probable error of a series of experiments carried out on the same day with the same antigen and serum, provided a measure of the accuracy of the methods. The probable error was always less than 10% with an average of 6.4%. Determinations which showed a greater variation between duplicates than this were disregarded. Similar calculations for the same series carried out on six different days with six different samples of pooled complement showed, as would be expected, a greater probable error because of the further variables introduced. The maximum probable error was 17.3%, the minimum 4.3% and the average 10.9%; the greatest percentage error was of course in the normal serum systems for here the variations bulk proportionately larger in view of the lesser total amount of complement fixed. The specific fixation is subject to the least degree of error because it is least dependent upon the quality of the complement.

Experimental Results

(*a*) *Preliminary Studies with Standard S and R Antigens and Antisera*

The preliminary studies of the antigenic activity of the S and R types of tubercle bacilli have been carried out with three strains of this organism: No. 599, an avirulent bovine strain; No. 54, an avirulent; and No. 13, a virulent human strain. The history and characteristics of these cultures were given in the second paper in this series (20). It was shown that No. 599 and No. 54 produce the typical R type of colony, form massive pellicles on liquid medium, agglutinate at relatively high acidity and show lack of virulence for guinea pigs: whereas, No. 13 produces the S type of colony and a veil-like pellicle on liquid medium, agglutinates at a relatively high acidity and exhibits virulence for guinea pigs.

In Table I are reported the results of a typical series of experiments carried out upon one day. Three antigens were compared in the presence of one immune serum and one normal rabbit serum, the degree of fixation being about the same in each case. The values have been plotted in Fig. I. A somewhat S-shaped curve similar to that reported by others who have studied the hemolytic reaction quantitatively (Brooks (7), Coulter (9) and others) was obtained.

The total fixation for S antigen No. 13 in the presence of R No. 599 antiserum at 40, 50 and 60% inhibition of hemolysis was 0.0080, 0.0072 and 0.0061 cc.; for R antigen No. 54, 0.0072, 0.0065 and 0.0055 cc.; and for antigen No. 599, 0.0078, 0.0069 and 0.0058 cc. The specific fixation, *i.e.*, the total fixation at these points less the amount of complement fixed in the normal system at the same points, was for the same antigens, 0.0063, 0.0057 and 0.0048; 0.0053, 0.0050 and 0.0043; 0.0060, 0.0052 and 0.0043 cc. respectively. If we compare the specific activity of the heterologous antigens at these three points with that of the homologous No. 599 antigen, we obtain these average ratios; for

the No. 13, an average of 1.08 to 1; for the No. 54 antigen the relationship is very similar, 0.95 to 1. The three antigens were apparently reacting with the same type of antibody in the antiserum, indicating some common antigenic property in S and R extracts. Each was apparently being used in sufficient excess to react with all the antibody present.

TABLE I

A COMPARISON OF THE PERCENTAGE INHIBITION OF HEMOLYSIS OBTAINED WITH ANTIGENS No. 13 (S), No. 54 (R) AND No. 599 (R) IN THE PRESENCE OF NORMAL AND IMMUNE RABBIT SERUM

Amount of complement cc.	Normal serum						Immune serum (No. 599)					
	No. 13 Antigen		No. 54 Antigen		No. 599 Antigen		No. 13 Antigen		No. 54 Antigen		No. 599 Antigen	
	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
0.0131	0	0	0	0	0	0	0	0	0	0	0	0
0.0118	0	0	0	0	0	0	10	5	0	0	10	10
0.0106	0	0	0	0	0	0	20	15	10	5	20	15
0.0096	0	0	0	0	0	0	30	30	15	15	30	25
0.0084	0	0	0	0	0	0	35	40	25	30	35	35
0.0076	0	0	0	0	0	0	40	50	30	40	40	50
0.0068	0	0	0	0	0	0	50	55	40	50	45	55
0.0061	0	0	0	0	0	0	60	60	50	60	50	60
0.0055	0	0	0	0	0	0	63	68	55	65	60	68
0.0050	0	0	0	0	0	0	70	75	60	70	65	70
0.0045	0	0	0	0	0	0	80	80	65	80	70	73
0.0040	0	0	0	0	0	0	90	90	70	83	73	78
0.0036	0	0	0	0	0	0	93	93	80	85	85	80
0.0032	0	0	0	0	0	0	95	98	85	93	93	90
0.0029	0	0	0	0	0	0	100	100	93	95	95	93
0.0026	0	5	10	5	5	5	100	100	95	100	98	98
0.0023	10	10	15	15	15	15	100	100	98	100	100	100
0.0021	15	25	30	25	25	20	100	100	100	100	100	100
0.0019	25	30	40	40	40	35	100	100	100	100	100	100
0.0017	40	40	45	45	50	50	100	100	100	100	100	100
0.0015	50	50	50	50	65	60	100	100	100	100	100	100
0.0013	60	60	55	60	70	70	100	100	100	100	100	100
0.0012	70	75	60	75	80	75	100	100	100	100	100	100
0.0011	80	80	75	80	90	80	100	100	100	100	100	100
0.0010	95	98	85	90	95	95	100	100	100	100	100	100

The results of a series of such tests have been assembled in Table II, the probable error of each mean value being inserted at the foot of the table. It may be seen, as was pointed out previously, that the per cent probable error on the specific fixation values is considerably less than that on the mean total amount of complement fixed. For this reason, in later experiments, specific fixation values only have been considered.

In Table III the results of similar series with different antigens and antisera have been averaged, each value representing the mean amount of complement fixed on five or more different days. The probable error of this mean value was calculated and has been inserted in the table below the mean values themselves. Although the slight differences in the amount of fixation by the various

TABLE II

TOTAL AND SPECIFIC FIXATION OF COMPLEMENT BY AN "R" BOVINE ANTIGEN No. 599 IN THE PRESENCE OF ITS HOMOLOGOUS IMMUNE SERUM

Date tested 1930	Total fixation of complement at			Specific fixation of complement at		
	40%	50%	60%	40%	50%	60%
May 7	0.0068 0.0072 0.0068	0.0066 0.0065 0.0065	0.0055 0.0060	0.0051	0.0047	0.0047
May 9	0.0076 0.0083 0.0078	0.0064 0.0070 0.0069	0.0061 0.0065	0.0054	0.0048	0.0046
May 14	0.0071 0.0071	0.0063 0.0065	0.0052 0.0058	0.0053	0.0053	0.0038
May 22	0.0080 0.0083	0.0076 0.0080	0.0064 0.0076	0.0054	0.0052	0.0050
May 29	0.0071 0.0072	0.0063 0.0063	0.0055 0.0054	0.0055	0.0053	0.0050
Average	0.0073	0.0068	0.0059	0.0053	0.0049	0.0047
Probable error	0.00079	0.00076	0.00067	0.0009	0.00028	0.00020

TABLE III

A COMPARISON OF THE ANTIGENIC ACTIVITY OF ANTIGENS No. 13, 54 AND 599 IN THE PRESENCE OF No. 13, 54 AND 599 ANTISERA

Antiserum No.	Antigen No.	Total fixation at			Specific fixation at		
		40%	50%	60%	40%	50%	60%
13 (S)	13 (S)	0.0113 ±0.0014	0.0104 ±0.0014	0.0095 ±0.0013	0.0089 ±0.0003	0.0083 ±0.0006	0.0074 ±0.0005
13 (S)	54 (R)	0.0063 ±0.0006	0.0053 ±0.0003	0.0046 ±0.0002	0.0038 ±0.0004	0.0032 ±0.0004	0.0030 ±0.0004
13 (S)	599 (R)	0.0060 ±0.0007	0.0048 ±0.0005	0.0044 ±0.0005	0.0036 ±0.0004	0.0033 ±0.0004	0.0031 ±0.0004
54 (R)	13 (S)	0.0079 ±0.0006	0.0068 ±0.0006	0.0062 ±0 0004	0.0054 ±0.0005	0.0046 ±0.0004	0.0044 ±0.0004
54 (R)	54 (R)	0.0058 ±0.0006	0.0053 ±0.0004	0.0048 ±0.0005	0.0034 ±0.0003	0.0033 ±0.0002	0 0030 ±0 0002
54 (R)	599 (R)	0.0064 ±0.0009	0.0058 ±0.0008	0 0053 ±0.0007	0.0038 ±0.0003	0 0035 ±0.0004	0 0032 ±0 0004
599 (R)	13 (S)	0.0074 ±0.0013	0.0065 ±0.0007	0 0039 ±0 0006	0.0048 ±0 0008	0 0045 ±0 0006	0 0041 ±0 0005
599 (R)	54 (R)	0.0066 ±0.0007	0.0061 ±0.0007	0 0056 ±0 0007	0 0038 ±0 0002	0 0035 ±0 0002	0 0034 ±0 0003
599 (R)	599 (R)	0.0068 ±0.0005	0.0061 ±0.0004	0 0054 ±0 0003	0 0043 ±0 0001	0 0038 ±0 0003	0 0037 ±0 0002

antigens in the presence of the No. 599 and No. 54 antisera fell within the range of the probable error, in the presence of No. 13 antiserum, the No. 13 antigen fixed about twice as much complement as the R antigens, a difference equivalent to more than four times the probable error on the total fixation and at least eight times that on the specific fixation. In the presence of the standard S No. 13 antiserum, practically the same amount of fixation was secured with a 1 in 30 dilution of either antigen No. 54 or No. 599 as was given in a 1 in 20. In a dilution of 1 in 40 the fixation was decreased slightly. Antigen No. 599 diluted 1 in 30 and No. 54 diluted 1 in 25 contained sufficient antigenic substance to fix specifically about twice the amount of complement in the presence of two units of S antiserum that they fixed in the presence of one unit. The differences in the behavior of the R antigens would appear therefore to be of a qualitative rather than quantitative nature.

TABLE IV

RATIO OF THE SPECIFIC FIXATION OF COMPLEMENT BY ANTIGENS NO. 599, 54 AND 13 IN THE PRESENCE OF NO. 13, 54 AND 599 ANTISERA, TO THE SPECIFIC FIXATION IN THE PRESENCE OF THE NO. 13 ANTIGEN IN THE PRESENCE OF THE SAME ANTISERUM

Ratio at	Antiserum No.	Antigen No. 13 (S)	Antigen No. 54 (R)	Antigen No. 599 (R)
40% Inhibition of hemolysis	13 (S)	1:1	0.43:1	0.40:1
50% Inhibition of hemolysis	13 (S)	1:1	0.40:1	0.40:1
60% Inhibition of hemolysis	13 (S)	1:1	0.41:1	0.42:1
40% Inhibition of hemolysis	54 (R)	1:1	0.63:1	0.71:1
50% Inhibition of hemolysis	54 (R)	1:1	0.72:1	0.76:1
60% Inhibition of hemolysis	54 (R)	1:1	0.69:1	0.73:1
40% Inhibition of hemolysis	599 (R)	1:1	0.79:1	0.88:1
50% Inhibition of hemolysis	599 (R)	1:1	0.78:1	0.85:1
60% Inhibition of hemolysis	599 (R)	1:1	0.82:1	0.90:1

The similarity in the behavior of the S and R antigens in the presence of R antiserum, and their differences in the presence of S antiserum, is more apparent however from the ratios shown in Table IV.

(b) Study of other Extracts and Antisera Prepared from the Same Strains

These preliminary experiments seemed to show that extracts made from R organisms, whether of bovine or human origin, lacked something present in S preparations. Antisera prepared against S organisms contained not only those antibodies which reacted with R preparations, but also other antibodies not detected by R antigens, which reacted with an S antigen. The antibodies in the R antisera on the other hand, had their counterpart in both S and R antigens. The importance of using an S antigen in the titration of S antisera was therefore evident.

Extracts subsequently prepared from these same R strains behaved in the presence of the same antisera in a manner essentially similar to the first preparations. Certain minor quantitative or even qualitative differences between different preparations were to be expected; differences dependent upon the efficiency of extraction, the colloidal dispersion of the antigenic materials and the efficiency of the dialysis. As is shown in Table V, in the presence of two new S antisera, No. 6803 and 6804 respectively, the various R antigens were approximately one-third and one-half as efficient as the S antigens. These antisera would also seem to contain two types of antibodies, one of which is S-specific.

TABLE V

COMPARISON OF THE ACTIVITY OF AN S AND VARIOUS R ANTIGENS IN THE PRESENCE OF TWO S ANTISERA, USING THE SPECIFIC FIXATION THE STANDARD S (13) ANTIGEN AS THE UNIT OF COMPARISON

Antigen No.	Anti-serum (S) No.	Specific fixation at			Ratio of specific fixation to that with the standard S antigen		
		40%	50%	60%	40%	50 %	60%
Standard S	6803	0.0137	0.0116	0.0111	1.1	1.1	1.1
599–1–R	6803	0.0053	0.0049	0.0046	0.39:1	0.42:1	0.41:1
599–2–R	6803	0.0053	0.0047	0.0044	0.39:1	0.41:1	0.39:1
599–3–R	6803	0.0038	0.0037	0.0035	0.27:1	0.31:1	0.31:1
54–1–R	6803	0.0033	0.0028	0.0026	0.27:1	0.25:1	0.23:1
54–2–R	6803	0.0046	0.0040	0.0039	0.33:1	0.34:1	0.35:1
Standard S	6804	0.0060	0.0058	0.0056	1.1	1.1	1.1
599–1–R	6804	0.0038	0.0036	0.0032	0.53:1	0.62:1	0.57:1
599–2–R	6804	0.0033	0.0028	0.0022	0.55:1	0.49:1	0.40:1
599–3–R	6804	0.0038	0.0027	0.0023	0.63:1	0.48:1	0.41:1
54–1–R	6804	0.0040	0.0032	0.0029	0.67:1	0.55:1	0.52:1
54–2–R	6804	0.0032	0.0027	0.0022	0.54:1	0.46:1	0.41:1

(*c*) *Behavior of Antigens Prepared from other Cultures of Mammalian Tubercle Bacilli*

Other known S and R cultures of tubercle bacilli were used in the preparation of antigens and antisera. An aqueous extract of the virulent bovine strain, No. 56, previously studied (20), was found to behave in the presence of R antiserum and with both the standard S No. 13 and its own homologous antiserum, in a manner similar to the S antigens previously studied (Table VI). The No. 56 antisera also contained antibodies with which R antigens, whether of human or bovine origin, did not react.

Several strains of B.C.G. have been maintained in this laboratory for some time. One of these, the Paris strain, has for two years consistently produced only R colonies and has failed to produce progressive tuberculosis in guinea pigs. Another, the Perif. strain isolated from a culture of B.C.G. obtained from Dr. Petroff, is composed of almost pure S types and leads in guinea pigs to a generalized tuberculosis. Aqueous extracts and antisera were prepared from both of these strains.

In the presence of standard R antiserum, the two B.C.G. antigens were only some 40 to 50% as efficient as the standard S and R antigens (Table VI). The reason for this is probably an unsatisfactory extraction since the extracts were water clear, unless indeed B.C.G. cultures are low in antigenic properties. Notwithstanding the relative inefficiency of antigens, there appeared to be a significant difference in the behavior of the Perif. or S antigens and the Paris or R antigens, in the presence of the standard S antiserum, the former being

TABLE VI

A COMPARISON OF THE ACTIVITY OF VARIOUS S AND R ANTIGENS IN THE PRESENCE OF S AND R ANTISERA WITH THAT OF THE STANDARD S AND R ANTIGENS

Antigen	Specific fixation at			Ratio of specific fixation to that of	
	40%	50%	60%	Standard R	Standard S
(a) 599 Antiserum (R)					
56–1	0.0078	0.0077	0.0076	0.80:1	0.97:1
B.C.G.–Perif.	0.0050	0.0046	0.0041	0.48:1	0.58:1
B.C.G.–Paris	0.0049	0.0039	0.0030	0.41:1	0.50:1
(b) 56 Antiserum (S)					
56–1	0.0080	0.0079	0.0077	1.49:1	1.02:1
54–1	0.0050	0.0047	0.0046	0.98:1	0.62:1
B.C.G.–Paris	0.0030	0.0027	0.0025	0.52:1	0.36:1
B.C.G.–Perif.	0.0047	0.0045	0.0044	1.11:1	0.77:1
(c) B.C.G.–Paris antiserum (R)					
13–2	0.0078	0.0076	0.0070	0.93:1	0.81:1
56–2	0.0073	0.0071	0.0060	0.84:1	0.73:1
B.C.G.–Paris	0.0098	0.0090	0.0088	1.16:1	0.89:1
B.C.G.–Perif.	0.0088	0.0087	0.0073	1.02:1	0.89:1
(d) B.C.G.–Perit. antiserum (S)					
B.C.G.–Perif.	0.0051	0.0044	0.0040	1.41:1	0.67:1
B.C.G.–Paris	0.0019	0.0017	0.0016	0.56:1	0.26:1

nearly twice as active as the latter. Whereas, in the presence of R antiserum, the efficiency ratio of the Perif antigen to the standard R antigen was 0.48 to 1, in the presence of the S antiserum, the ratio became 1.11 to 1, a difference made up doubtless by the presence of certain S antigenic substances in the Perif. preparations. Such a supposition receives further confirmation in the observation that the efficiency ratios of this antigen to the standard S and R antigens were also increased in the presence of S antisera. The Paris antigen behaves in all respects like other R antigens of low titre. These findings, although far from complete, suggest that the change in colony form and acquisition of virulence in this one strain of B.C.G. Perif has been accompanied by a gain in specific antigenic substances. The undissociated strain of B.C.G. appears to resemble other R strains in antigenic characteristics.

As may be seen from Table VII, these B.C.G. strains produce antisera which react in a manner similar to antisera prepared against other S and R tubercle bacilli when tested with standard S and R antigens. The results of tests of various additional S and R antisera with these standard antigens further support the conclusions previously reached.

(*d*) *Antigenic Relationship with S and R Types of Other Acid-fast Species*

Several stains of other acid-fast species have been dissociated (18). Comparative tests were made with one strain of *Myco. leprae*, the L-65 or Duval strain obtained from the American Type Culture Collection, from which very definite S and R types had been isolated, and with Petroff's avian S and R cultures (17, 20).

TABLE VII

A COMPARISON OF THE REACTIVITY OF THE STANDARD R AND S ANTIGENS IN THE PRESENCE OF VARIOUS ANTISERA

Antiserum	Antigen	Specific fixation at			Average ratio S:R	Average ratio R:S
		40%	50%	60%		
(a) S antiserum						
6805 (56)	St. S	0.0089	0.0075	0.0066	1.46:1	—
6805 (56)	St. R	0.0056	0.0052	0.0050	—	0.67:1
956 (13)	St. S	0.0059	0.0053	0.0048	1.77:1	—
956 (13)	St. R	0.0031	0.0030	0.0029	—	0.56:1
986 (13)	St. S	0.0050	0.0045	0.0044	1.98:1	—
986 (13)	St. R	0.0025	0.0023	0.0022	—	0.50:1
B.C.G.–Perif.	St. S	0.0071	0.0064	0.0062	2.05:1	—
B.C.G.–Perif.	St. R	0.0039	0.0030	0.0027	—	0.50:1
6803 (13)	St. S	0.0060	0.0038	0.0056	1.41:1	—
6803 (13)	St. R	0.0038	0.0032	0.0032	—	0.60:1
(b) R antiserum						
6801 (599)	St. S	0.0080	0.0079	0.0078	0.82:1	—
6801 (599)	St. R	0.0100	0.0099	0.0090	—	1.22:1
958 (599)	St. S	0.0093	0.0092	0.0088	0.97:1	—
958 (599)	St. R	0.0095	0.0094	0.0093	—	1.03:1
980 (599)	St. S	0.0098	0.0093	0.0088	0.78:1	—
980 (599)	St. R	0.0132	0.0121	0.0108	—	1.26:1
970 (54)	St. S	0.0070	0.0068	0.0067	0.76:1	—
970 (54)	St. R	0.0096	0.0090	0.0083	—	1.33:1
B.C.G.–Paris	St. S	0.0099	0.0090	0.0087	1.16:1	—
B.C.G.–Paris	St. R	0.0085	0.0080	0.0077	—	0.87:1

Extracts of the S and R leprae cultures, after preliminary tests, were compared with the standard S and R tubercle bacillus antigens in the presence of various S and R tuberculous antisera (Table VIII). The S leprae extracts were always definitely inferior to the R extracts of the same organisms. Part at least of this difference appeared to be due to incomplete extraction, although the possibility that these S organisms contained less of the antigenic substances common to the two species of acid-fast bacteria than the R types of the same, could not be disregarded.

The data in Table VIII would also seem to indicate that antisera prepared against the R type of tubercle bacillus contained a higher percentage of antibodies reacting with the related acid-fast species, *Myco. leprae*, than antisera prepared against the S types of tubercle bacilli.

Aqueous extracts made from Petroff's avian S and R cultures, when tested in the presence of various S and R human and bovine tuberculous antisera, were found to behave in a very similar manner to the leprae antigens just described, only a fraction of the antibodies contained in these sera being fixed. That this fixation was due, in part at least, to the presence of group antibody was shown by the fact that they also reacted to some extent with the leprae antisera. The avian R antigens reacted somewhat more strongly than the S with the tuberculous antisera, another finding which is in agreement with the observations made with leprae antigens.

TABLE VIII

A COMPARISON OF THE AVERAGE SPECIFIC FIXATION OF ANTIGENS PREPARED FROM S AND R CULTURES OF LEPRAE BACILLI IN THE PRESENCE OF VARIOUS ANTISERA, WITH THE SPECIFIC FIXATION OF THE STANDARD S AND R TUBERCLE BACILLUS ANTIGENS IN THE SAME SYSTEM

Antiserum	Leprae S: St. S	Leprae S: St. S	Leprae R: St. S	Lepare R: St. S
(a) S antisera				
100 (13)	0.13:1	0.34:1	0.35:1	0.69:1
6804 (56)	0.23:1	0.35:1	0.33:1	0.48:1
6803 (13)	0.29:1	0.49:1	0.30:1	0.43:1
956 (13)	0.22:1	0.42:1	0.39:1	0.65:1
983 (13)	0.21:1	0.43:1	0.42:1	0.76:1
B.C.G.–Perif.	0.17:1	0.38:1	0.27:1	0.58:1
Average	0.20:1	0.40:1	0.34:1	0.60:1
(b) R antisera				
6801 (599)	0.32:1	0.39:1	0.65:1	0.52:1
980 (599)	0.31:1	0.24:1	0.62:1	0.48:1
958 (599)	0.14:1	0.11:1	0.46:1	0.44:1
970 (500)	0.05:1	0.04:1	0.53:1	0.41:1
Average	0.21:1	0.19:1	0.57:1	0.46:1

(e) Suggestions as to the Type of Antibodies Constituting S and R Antisera

In Table IX are summarized some of the data from the preceding tables and also data from several experiments not here recorded. The tuberculous immune sera have been divided into two groups, those prepared against a virulent strain of tubercle bacillus showing typical S-like colonies, and those prepared against attenuated strains characterized by R-like colonies. These sera have been tested in the presence of various S and R antigens, but for conciseness, only the standard S and R antigens of the tubercle bacillus and of the R antigens of the leprosy bacillus are dealt with.

In an S antiserum, an S antigen present in some excess, may be considered to fix 100% of the antibodies; the other two antigens, various percentages of this amount as determined by the ratios previously shown. Similarly in an R antiserum, the standard R antigen would fix 100% of the antibodies, the other antigens different proportions of this total amount.

The existence in an S antiserum of three types of antibodies may be postulated: (a) those specific for the S type only; (b) those reacting also with the R type, a species antibody; and (c) those which react in addition with related acid-fast species, a group antibody. Corresponding to these antibodies, there would be present in the S organisms, the type-specific or S-specific, the species-specific and group antigenic substances.

TABLE IX

PERCENTAGE FIXATION WITH VARIOUS ANTIGENS IN THE PRESENCE OF VARIOUS R AND S TUBERCULOUS IMMUNE SERA AS COMPARED WITH THE SPECIFIC FIXATION WITH THE HOMOLOGOUS R OR S STANDARD ANTIGEN

Antisera	Per cent of antibodies fixed by			Antisera	Per cent of antibodies fixed by		
	Standard S	Standard R	Leprae R		Standard S	Standard R	Leprae R
(a) S (T.B.) antisera				(b) R (T.B.) antisera			
100	100	41	35	6801	82	100	52
6803	100	41	—	6802	114	100	—
6804	100	67	33	970	76	100	41
956	100	56	39	980	78	100	48
8866	100	50	62	958	97	100	44
Perif.	100	50	27	Paris	110	100	52
6805	100	60	48	5799	96	100	44
6806	100	57	36	6800	90	100	50
6807	100	47	46	Average	93	100	47
Average	100	52	41				

Using the values in Table IX, an average S antituberculous serum may be visualized as consisting of 48% S-specific, 11% species-specific and 31% group-specific antibodies. In referring to the S-specific antibodies as type-specific, it should be noted, as has been done in a previous section, that bovine and human stains seem to elicit the same type-specific antibodies, at least as denoted by the complement fixation reaction, but that these are different from the type-specific factors of the avian strains.

By similar reasoning, an average R antiserum would contain 7% R-specific, 46% species-specific and 47% group antibodies, giving about the same proportion of group antibodies in both S and R antisera. With the change from S to R, there has been a substitution of species-specific factors for the type-specific groups. Whether the 7% R-specific substances has any significance is doubtful because such a low value falls within the range of the probable error of the specific fixation values.

Discussion

Investigation of the antigenic properties of S and R types of various species has indicated that the S and R change in those species in which the S form is the normal one, involves a loss in specificity, the resultant R type being more closely related to the R of other strains than to its homologous S.

The change from S to R in tubercle bacilli also seems to involve a loss in some S-specific substance, an antigenic change of the type occurring in the dissociation of the pneumococci and streptococci, but whether this change is related to the loss of carbohydrate substance, as in the case of the pneumococcus has not been determined. In addition to the S-specific and species antigen, there also appears to be a group antigen in the tubercle bacillus, an antigen which reacts with antisera prepared against other acid-fast species. This reaction is reciprocal, antisera prepared with vaccines of either S or R types of tubercle bacilli containing antibodies which react with closely related acid-fast bacteria. That part of this group reaction may depend upon a non-specific reaction with certain lipoids contained in extracts of *Myco. leprae* or avian tubercle bacilli, which like all other acid-fast organisms are high in lipoidal content, is suggested tentatively.

The observation that R antigens detect only some 50% of the antibodies in sera prepared against virulent organisms, in fact that they are only a little better than extracts prepared from related acid-fast species, seems of importance from a diagnostic standpoint. It is just possible that some of the unsatisfactory results which have obtained with the complement-fixation test in the diagnosis of tuberculous infection, may have been due in part to the use of an antigen prepared from cultures which were not fully virulent. Although it is not known in how far tuberculous antibodies detected by the complement-fixation reaction are related to immunity in the infected animal, it does not seem unjustifiable to wonder in how far these differences in antigenic behavior may affect the value of R cultures as immunizing agents.

Summary

By a quantitative technique, it has been shown that the S to R change in human and bovine tubercle bacilli is accompanied by a loss in specific antigenic activity in the complement-fixation reaction. All preparations from attenuated cultures in which the R colony has been shown to be predominant, have been found to be much inferior as antigens to similar preparations made from cultures composed of S types.

Antisera prepared from virulent S organisms have been shown to contain S-specific antibodies, in addition to species antibodies which also react with R antigens, and group antibodies detected by preparations made from related acid-fast species. The S-specific antigenic substances in virulent human and virulent bovine cultures appeared to be qualitatively similar.

Antisera prepared against R organisms have been found to contain species and group antibodies, the former being present in a higher proportion than in S antisera.

Acknowledgment

The author is indebted to Dr. G. B. Reed of the Department of Bacteriology, Queen's University, and to Dr. W. L. Holman of the Department of Pathology and Bacteriology of the University of Toronto, for helpful suggestions and criticisms.

References

1. ARKWRIGHT, J. A. J. Path. Bact. 31: 665-670. 1928.
2. ARKWRIGHT, J. A. and PITT, R. M. J. Path. Bact. 32: 229-246. 1929.
3. ARONSON, J. D. and WHITNEY, C. E. J. Infectious Diseases, 47: 30-55. 1930.
4. BABÈS, B. and BUSILIA. Compt. rend. soc. biol. 68: 181. 1910.
5. BEGBIE, R. S. Edinburgh Med. J. 37: 187-191. 1930.
6. BORDET, J. and GENGOU, O. Compt. rend. soc. biol. 73: 122. 1903.
7. BROOKS, S. C. J. Med. Research, 41: 399. 1920.
8. COOKE, J. V. J. Infectious Diseases, 25: 452-473. 1919.
9 COULTER, C. B. J. Gen. Physiol. 10: 541-550. 1927.
10. COULTHARD, H. L. J. Path. Bact. 26: 350-381. 1923.
11. GRIFFITH, F. A system of bacteriology, v. 5: 150. 1930.
12. HARRIS, W. H. and LANFORD, J. A. J. Infectious Diseases, 13: 301-308. 1913.
13. KIRCHNER, O. Beit. Klin. Tuberk. 66: 584-587. 1927.
14. MALTANER, E. J. Immunol. 1931. (In press).
15. PETROFF, S. A., BRANCH, A. and STEENKEN, W. Proc. Soc. Exptl. Biol. Med. 25: 14-16. 1927.
16. PETROFF, S. A., BRANCH, A. and STEENKEN, W. Am. Rev. Tuberculosis, 19: 9-39. 1929.
17. PETROFF, S. A. and STEENKEN, W. J. Exptl. Med. 51: 831-845. 1930.
18. REED, G. B. J. Bacteriol. (In press).
19. REED, G. B. and RICE, C. E. Can. J. Research, 4: 389-398. 1931.
20. REED, G. B. and RICE, C. E. Can. J. Research, 5: 111-121. 1931.
21. SCHEFF, L. D. Am. Rev. Tuberculosis, 12: 45-48. 1925.
22. SHIBATA, J. Z. Immunitäts. 63: 358-370. 1929.
23. SMITH, J. Lancet, 203 (2): 13-14. 1922.
24. TODD, E. W. and LANCEFIELD, R. C. J. Exptl. Med. 48: 751-767. 1928.
25. WADSWORTH, A. B. Standard methods of the Division of Laboratories and Research of the New York Department of Health, Williams and Wilkins, Baltimore, 1927.
26. WADSWORTH, A., MALTANER, F. and MALTANER, E. J. Immunol. 10: 241-332. 1925.
27. WHITE, P. B. A system of bacteriology. v. 4. 1929.